PHD

Design and synthesis of isoform selective inhibitors of nitric oxide synthase for cancer therapy

Goodyer, Claire Louise Margaret

Award date:
2003

Awarding institution:
University of Bath

Design and Synthesis of Isoform Selective Inhibitors of Nitric Oxide Synthase for Cancer Therapy

submitted by

Claire Louise Margaret Goodyer BSc (Hons)

for the degree of PhD

of the University of Bath

2003

.....CMGoodyer.....

...17th January 2003

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

This thesis describes the synthesis and evaluation of compounds designed as isoform-selective nitric oxide synthase inhibitors, carrying a 4,5-dihydrothiazole or a thiourea. Structural features of the selective iNOS inhibitor 1400W were incorporated into the design.

Human tumours have high levels of NO, the main source being iNOS. NO causes increased tumour blood flow. Selective iNOS inhibition should cause collapse of the tumour vasculature, an antitumour event, but will also increase tumour hypoxia and potentiate bioreductively activated drugs.

The principal target compounds were N-benzyl and N-phenylthioureas, 2-benzylamino-4,5-dihydrothiazoles and 2-phenylamino-4,5-dihydrothiazoles. Isothiocyanates reacted with ammonia giving thioureas. Reaction of 3- and 4-methoxybenzylamines with 2-methylthio-4,5-dihydrothiazole at 180°C gave the corresponding dihydrothiazoles. Other dihydrothiazoles were synthesised by acid-catalysed cyclisation of N'-(2-hydroxylethyl)thioureas. N^{ε}-Aminothiocarbonyl-L-lysine and its methyl ester were synthesised similarly. N^{ε}-(4,5-Dihydrothiazol-2-yl)lysine was synthesised via the N'(2-hydroxyethyl)thiourea intermediate. The benzene rings carried various groups in meta or para positions to explore the structure-activity relationships.

The five most potent targets were analysed for time-dependent kinetics, giving an optimum pre-incubation time of fifteen minutes. N^{ε}-Aminothiocarbonyl-L-lysine and its methyl ester were potent, N^{ε}-aminothiocarbonyl-L-lysine methyl ester displaying four-fold selectivity for cNOS. Both compounds were more potent than the known inhibitor thiocitrulline against the cNOS isoform and equivalent to L-NMMA against the iNOS isoform. The dihydrothiazoles were the most potent NOS inhibitors. 2-(3-(Aminomethyl)phenylamino)-4,5-dihydrothiazole was more potent than the known inhibitor 7NI but showed no isoform selectivity. N-(3-aminomethylphenyl)thiourea showed five-fold selectivity for the cNOS isoform, and with $IC_{50} = 54$ μM. The most potent and selective aromatic targets carried CH_2NH_2,

with the most selective being meta-substituted, which provides an optimum size for binding to NOS.

N-(2-Hydroxyethyl)-N'-(3-methoxyphenylmethyl)thiourea stimulated iNOS without pre-incubation. After ten minutes pre-incubation, it was weakly inhibitory. Other thioureas exhibited similar properties. These compounds may bind at more than one site, one site being stimulatory and the other inhibitory.

Acknowledgments

I would like to thank my supervisor Dr Mike Threadgill for his unlimited support, encouragement and advice throughout my research project. My thanks go to Dr Edwin Chinje for his help in teaching me the biological assays involved in this project and for extracting the enzymes for me. Special thanks are also due to Dr Mohammed Jaffar for his help and advice with all aspects of my thesis.

Thanks to Dr Steve Black and Mr David Wood for the provision of NMR spectra and to Mr Chris Cryer for the mass spectra data. To Mr Alan Carver for the microanalysis and also to Dr Andy Thompson for help in compiling the SAR pictures.

I would like to thank my colleagues in the research group Esther, Christian, Ghadeer, Mervat and especially Ifat, Christophe and Sandra who helped me learn many chemistry techniques in my first year.

I acknowledge and appreciate the financial support of the Association for International Cancer Research.

Finally I would like to express my greatest thanks to my family and friends who have offered endless support and encouragement to me throughout the last few years. My special thanks and love go to my Mum, Dad, brother Richard and Mike for believing in me.

Contents

List of Figures, Schemes, Tables and Graphs

Schemes

Tables

Abbreviations used

Ach	Acetylcholine
AIDS	Acquired immune deficiency syndrome
AMT	2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine
Aq	Aqueous
BH_4	Tetrahydropterin
Boc	1,1–Dimethylethoxycarbonyl
BSA	Bovine serum albumin
Bu^t	*tert*-Butyl
CaM	Calmodulin
Cbz	Phenylmethoxycarbonyl
COSY	Correlation spectroscopy
cGMP	Cyclic guanosine monophosphate
cNOS	Constitutive nitric oxide synthase
DNA	Deoxyribose nucleic acid
EDRF	Endothelium derived relaxing factor
EDTA	Ethylediaminetetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
EIT	Ethylisothiourea
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mono nucleotide
Glu	Glutamate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
h	Hour
HETCOR	Heteronuclear correlation
hiNOS	Human inducible nitric oxide synthase
HPLC	High performance liquid chromatography
HRE	Hypoxia response element
IL-2	Interleukin 2
iNOS	Inducible nitric oxide synthase

ITU	Isothiourea
L-NAME	N-nitro-L-arginine methyl ester
L-NIL	L-N^{ε}-(1-iminoethyl)lysine
L-NIO	L-N^{δ}-(1-iminoethyl)lysine
L-NMMA	N^{G}-monomethyl-L-arginine
L-NNA	N-nitro-L-arginine
LPS	Lipopolysaccharide
LTP	Long term potentiation
Min	minutes
7MI	7-Methoxyindazole
NADPH	Nicotinamide adenine diphosphate (reduced form)
7NI	7-Nitroindazole
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
NOH-Arg	N^{G}-hydroxy-L-arginine
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NOESY	Nuclear Overhauser enhancement spectroscopy
PARP	Poly (ADP-ribose) polymerase
PBITU	S,*S*'-(1.3-phenylenebis-(1,2-ethanediyl))bisisothiourea
PBS	Phosphate buffered saline
Pd/C	Palladium on activated charcoal (10%)
PDE5	Phosphodiesterase type 5
PKC	Protein kinase C
SAR	Structure activity relationship
SEM	Standard error of the mean
sGc-cGMP	Soluble guanylate cyclic GMP dependent pathway
SNT	S-nitrosothiols
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography

Introduction

What is Nitric Oxide?

The potentially toxic free radical nitric oxide (NO·) is currently one of the most studied molecules in biomedical sciences. The colourless inorganic gas is synthesised by mammals (Nathan 1992), birds (Lee *et al* 1994), invertebrate (Muller and Bicker 1994) and bacteria (Chen and Rossazza 1994). It was first discovered as endothelial derived relaxing factor (EDRF) in the vascular system, since then it has been identified in a variety of tissues and cells; including macrophages, artery, advential nerves and brain. NO also plays a role in plant disease resistance (Cho *et al* 1998).

NO is produced from L-arginine (**1**) and the reaction is catalysed by the enzyme nitric oxide synthase (NOS), of which there are three different isoforms. NO is both a chemical messenger and a cytotoxic agent. As a chemical messenger it acts as an initiator for biological pathways that are essential to life, such as blood flow (Kerwin *et al* 1995). The role NO plays as a cytotoxic agent can be both beneficial and harmful.

NO has been linked to many different disease states, some examples are: Alzheimer's disease, multiple sclerosis, AIDS-associated dementia, asthma, cancer, rheumatoid arthritis (Nathan 1997), Duchene's muscular dystrophy, sickle cell anaemia, sepsis, heart failure, erectile dysfunction, diabetes and atherosclerosis (Maxwell 2002).

Due to NO being related to many diseases the understanding of the biological effects of NO in healthy and disease states has great potential in therapeutic applications.

Physical Properties of NO

It is important to understand the chemical biology of NO in order to appreciate its biological roles. NO in the pure state under standard temperature and pressure is a gas. However NO acts as a dissolved nonelectrolyte in all its biological activities and so, in most biological conditions, NO is not a gas (Kerwin *et al* 1995). NO can

also have toxic effects by reacting with oxygen in an aerobic environment, producing toxic constituents of air pollution and cigarette smoke (Wink *et al* 1996b).

NO is an uncharged molecule. It is a diatomic radical containing eleven valence electrons, one of which makes the molecule paramagnetic. Its electron configuration is $(\sigma_1)^2\ (\sigma_2)^2\ (\sigma_3)^2\ (\pi)^4\ (\pi^*)^1$. The extra electron makes NO chemically highly reactive. The unpaired electron is in the antibonding molecular orbital and so is relatively easily removed to give the nitrosonium cation (NO^+). Conversely, the addition of an electron to this orbital forms NO^-. NO can act as a free radical, an electrophile and an oxidizing agent. NO is a relatively stable free radical which reacts readily with other free radicals such as dioxygen and superoxide (Stamler *et al* 1992).

Reactions with oxygen

NO reacts with O_2 in both the gas phase and aqueous solution to form NO_2.

$$2NO + O_2 \rightarrow 2NO_2$$

The reaction is second order with respect to NO and first order with respect to O_2. In the gas phase NO_2 will react further with NO_2 to form dinitrogen tetroxide (N_2O_4) or react with another NO to form dinitrogen trioxide (N_2O_3).

$$NO_2 + NO_2 \rightarrow N_2O_4$$

$$NO_2 + NO \rightarrow N_2O_3$$

These species are highly reactive and are considered donors of nitrosonium ion (NO^+). This ion can react with a variety of nucleophiles, including amines and thiols (Kerwin *et al* 1995).

Reaction with superoxide

The reaction of NO with superoxide is most likely to be the major route for NO metabolism. This reaction is very fast and produces the stable anion peroxynitrite; ($ONOO^-$).

$$NO + O_2^{\bullet-} \rightarrow ONOO^-$$

The stable nature of peroxynitrite allows it to diffuse through biological systems and across membranes before it reacts (Thomas 2000). Peroxynitrite is a potent oxidant which reacts with proteins, lipids and DNA. Peroxynitrite causes DNA damage by several mechanisms such as DNA base modifications and DNA single strand breakage (Szabo and Ohshima 1997).

Reaction with haemoglobin

NO has a much lower affinity for methemoglobin and other ferric (Fe^{3+}) compounds than for ferrous (Fe^{2+}) haemoglobin (Kerwin *et al* 1995). NO reacts with haem proteins such as haemoglobin, myoglobin, and cytochrome c to form the corresponding nitrosyl-haem protein adduct. NO reacts with oxyhaem to form stable compounds in the absence of oxygen, forming met Hb and NO_3^-. The first order dependence on NO concentration and the lack of a limiting rate at high NO concentration shows that NO reacts directly with bound O_2, without displacing it from the haem.

$$Hb\text{-}FeII\ (O_2) + NO \rightarrow Hb\text{-}FeIII + NO_3^-$$

This reaction limits the transport of NO in the blood, so that free NO does not have systemic effects.

Reaction with metals

NO can react with metalloproteins, such as intracellular cytochrome P-450 and cytochrome c oxidase. NOS has been found in mitochondria, so the competition between NO and O_2 binding to cytochrome c oxidase may be used to regulate respiration (Pfeiffer *et al* 1999). NO reacts with some (but not all) transition metal complexes to give metal-nitrosyl adducts. The rate and stability of these reactions depends on the valence state of the metal and the ligands in the coordination sphere (Wink *et al* 1996a).

Reaction with thiols

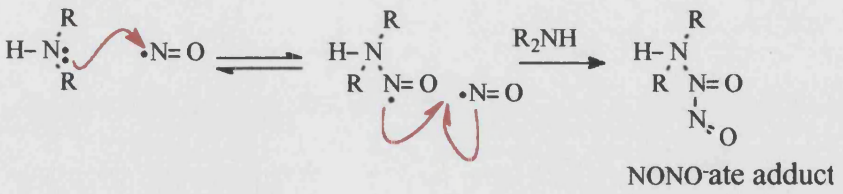
NO does not react directly with thiols to form S-nitrosothiols (SNTs or RS-NO). It is oxidised to the NO^+ species, which readily react with thiols and thiolates (RSH/RS^-), to form nitrosothiols (RS-NO). The major targets for the NO^+ species are metal- and thiol-containing proteins, in particular Zn-finger motifs within proteins (Stamler 1994).

Nitrous acid, alkylnitrites, nitrosyl halides, N_2O_3 and N_2O_4 all act as carriers for NO^+ (Pfeiffer *et al* 1999).

NO as an electrophile

Due to its electronic configuration, NO can act as an electrophile. NO reacts readily with primary and secondary amines to form adducts known as NONOates. The first step of the reaction involves NO reacting with the amine to form an NO complex. This acts as a radical and reacts with another NO molecule to form the NONO-ate adduct (scheme 1.1).

The NONO adduct is unstable and under aqueous conditions yields NO. These NONO-ate adducts may have therapeutic applications in conditions that results from impaired NO production e.g. angina (Thomas 2000).



Scheme 1.1: Mechanism to show the formation of a NONO-ate adduct

Nitric Oxide Synthases (NOS)

There are two major groups of NOS; constitutive NOS (cNOS) and inducible (iNOS). cNOS can be further sub-divided into endothelial and neuronal forms (eNOS and nNOS). These are the more common terminology used, however nNOS was the first enzyme to be isolated from brain tissue and is otherwise known as NOS-I. Similarly, iNOS was the second type to be isolated and is known as NOS-II and eNOS is also known as NOS-III. Soon after nNOS was cloned (Bredt *et al* 1991), the genes encoding all three enzymes were mapped on the human chromosome (Nathan and Xie 1994), with a 51-57% homology between the human forms (Alderton *et al* 2001). All three isoforms are found in a variety of tissues and a brief summary together with gene locations are given in table 1.1.

NOS isozyme		**Tissue expression**	**Human Chromosome location**
Neuronal	nNOS, ncNOS, Brain bNOS or type I	Neuronal cells Skeletal muscle Brain	12q24.2
Endothelial	eNOS, ecNOS or type III	Endothelial Epithelial Some neurons	7q35-36
Inducible	iNOS, macrophage mNOS or type II	Macrophages Hepatocytes Neutrophils	17q11.2

Table 1.1: Table illustrating the three isoforms of NOS and their gene location and tissue expression (Nathan and Xie 1994).

Both types are dependent on the reduced form of the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH). cNOS is Ca^{2+}/calmodulin-dependent; only the calcium-bound form of calmodulin (CaM) can bind and activate the enzyme. cNOS is unaffected by glucocorticoids and gives a short-lasting release of picomolar concentrations of NO. In contrast, iNOS is calcium-independent; this is because

iNOS already binds to CaM and is fully active at such low calcium concentrations that its activity can never be limited by calcium. iNOS is inhibited by glucocorticoids and sustains a long lasting release of nanomolar concentrations of NO (Pfeiffer *et al* 1999). The function of each of the isoforms will be discussed in turn later in the chapter.

Action of NO

Background

Evidence for mammalian nitric oxide synthesis was reported as early as 1916 but it was not until 1986 that Furchgott and Ignarro independently reported that EDRF and NO might be identical. At the same time, Ferid Murad was investigating the effectiveness of nitroglycerin as a treatment for heart attack. Nitroglycerin was shown to cause blood vessel relaxation once it had been converted to NO. NO relaxes muscles by instigating the formation of cyclic GMP in the same way that EDRF does (Snyder and Bredt 1992). Palmer *et al*, in 1987, showed that NO released from endothelial cells had identical chemical and biological properties to EDRF by stimulating the release of EDRF from endothelial cells and monitoring the relaxing effect on smooth muscle cells. At the same time the amount of NO released from the endothelium was monitored and was shown to fully account for the relaxation of muscle cells (Moncada *et al* 1991). These findings were later confirmed by Ignarro (Ignarro *et al* 1990).

In 1998, the Nobel Prize for physiology/medicine was awarded to Robert Furchgott, Louis Ignarro and Ferid Murad for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system (Physiology or Medicine for 1998).

The biological actions of NO are directly related to the cell type and tissue that produce it. NO is produced by a wide variety of cells and tissues such as endothelial cells, the central nervous system, peripheral nervous system, platelets and certain activated cells of the immune system. The actions of NO derived from the three different isoforms will be discussed separately.

eNOS

NO produced by cNOS acts as a chemical messenger and is unique because it relies on its redox reactivity to convey information to neighbouring cells, and is synthesised when required, unlike other chemical messengers which transmit information by changing their conformation and ability to bind to a receptor, are stored *in situ* and are released under specific stimulation (Thomas 2000). NO has a short biological life and, therefore, target cells must be close before NO is

metabolised to nitrate, nitrite and other reactive species. NO produced by cNOS isoforms is delivered to target cells in short bursts without damaging target cells and so is beneficial to biological processes.

eNOS is Ca^{2+}/CaM-dependent and is expressed in endothelial cells. Synthesis of NO by eNOS in vascular endothelial cells is stimulated by acetylcholine receptors (ACh). NO diffuses into smooth muscle cells of the blood vessel wall and elicits a cGMP-dependent relaxation, which results in an increased blood flow through the vessel (Gross 2001) (figure 1.1). The key role of eNOS is in maintaining a normal blood pressure (Knowles and Moncada 1994). Endothelial dysfunction is believed to be due to an increase in superoxide production within the endothelium. Superoxide reacts with NO to form peroxynitrite. This reduces the amount of NO diffusing into the smooth muscle cells making these cells less likely to relax, leading to an increase in blood pressure and endothelial damage (Thomas 2000).

NO inhibits platelet aggregation *via* the soluble guanylate cyclase cyclic GMP dependent pathway (sGc-cGMP). NO binds to a haem cofactor within the guanylate cyclase enzyme and activates it. This leads to intracellular accumulation of cGMP which then activates a cascade of intracellular enzymes to bring about biological effects. NO, together with prostacyclin, provides a defence against platelet aggregation and adhesion to the endothelium (Kerwin *et al* 1995).

The correct balance of NO produced by eNOS is extremely important. Excessive NO production within the blood vessel wall is thought to be the basis for conditions such as septic- and cytokine-induced circulatory shock. In these conditions, the sGc-cGMP pathway is excessively activated, which leads to high levels of NO and so contributes to profound vasodilatation and hypotension (Gross and Wolin 1995). However, if too little NO is produced, this can lead to conditions such as high blood pressure, angina and impotence. Recently, it has been shown that a reduction in the activity and synthesis of NO within the endothelium may contribute to the initiation and progression of atherosclerosis (Napoli and Ignarro 2001).

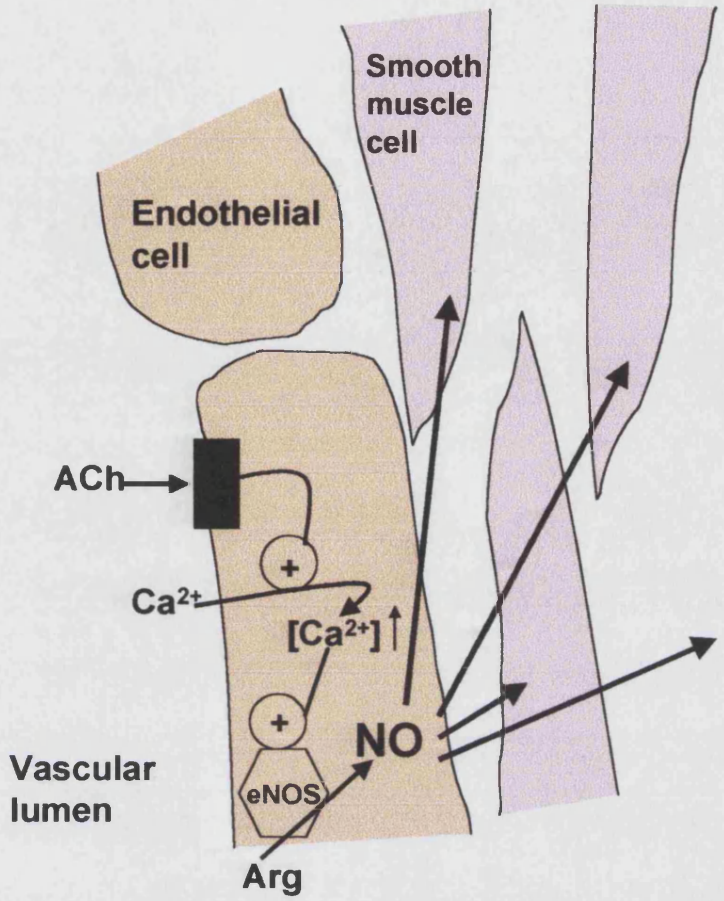


Figure 1.1: The pathway showing the production of NO by eNOS in a vascular endothelial cell which has been stimulated by Ach.

nNOS

nNOS is Ca^{2+}/CaM-dependent and is constitutively expressed at a high activity in the brain. It is expressed in postsynaptic terminals of neurons in the brain. Binding of neurotransmitter glutamate to receptors in the cell membrane causes calcium ions to influx and so activate the enzyme.

Activation of synapse begins with the impulse spreading to the presynaptic terminal, depolarising the terminal membrane and activating voltage gated calcium channels. This leads to exocytosis of synaptic vesicles and release of glutamate. Glutamate binds to receptors and calcium is carried into the cells. Here it acts as a second messenger and activates several kinds of kinases. Protein kinase C (PKC) is believed

to activate NO synthase, releasing NO. This diffuses to neighbouring terminals, including presynaptic terminals, where it activates guanylate cyclase to produce cGMP (figure 1.2). Memory function and learning is linked to a term known as long-term potentiation (LTP) which means that the synaptic response has greater amplitude and persists for a longer time. NO may provide the link which appears to be necessary for co-ordinating, the enhancement of both pre- and post-synaptic mechanisms which contribute to LTP (Shepherd 1994).

NO release from the pelvic nerve neurons located in the human corpus cavernosium is known to cause penile erection. NOS inhibitors have been shown to prevent this action while nitric oxide sources mimic the effect (Kerwin *et al* 1995). Nitric oxide activates guanylyl cyclase which forms GTP and activates cGMP which in turn cause penile erection, the activity is terminated by cGMP being converted to 5'-guanosine monophosphate by the action of phosphodiesterase type 5 (PDE5). The drug Viagra (Sildenafil) is a PDE5 inhibitor which compensates for the low levels of NO by allowing cGMP levels to accumulate (Thomas 2000).

Recently the over-expression of nNOS in circulating neutrophils has been found in patients with Parkinson's disease (Gatto *et al* 2000). Also nNOS levels are thought to be linked to migraine headaches. These headaches are believed to be a result of abnormal activity in large cerebral blood vessels and high levels of nNOS occur in the vasodilator nerves that supply the large cerebral blood vessels (Christopherson and Bredt 1997).

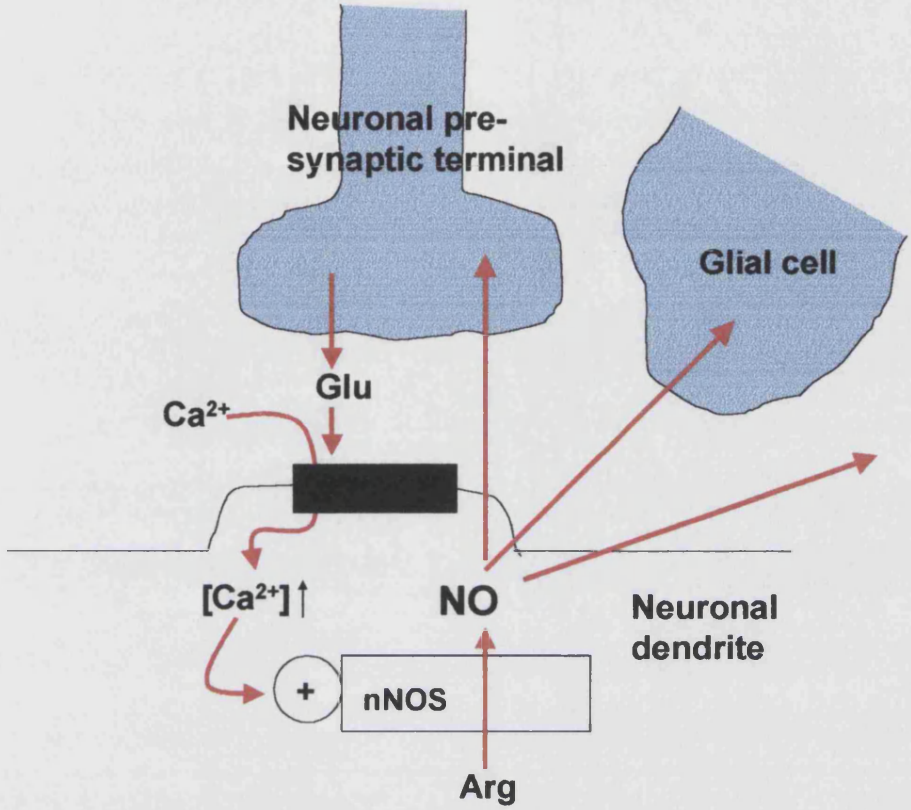


Figure 1.2: The pathway showing the production of NO by nNOS in a neuronal denrite which has been stimulated by glutamate (Glu).

iNOS

iNOS is Ca^{2+}/CaM-independent. This is because (as mentioned earlier) iNOS binds CaM already and is active at low Ca^{2+} concentration and so its activity is never limited by Ca^{2+}. iNOS is expressed in macrophages in response to cytokines and other stimuli associated with infection (figure 1.3). Expression typically begins 4-12 h after infection (Pfeiffer *et al* 1999).

NO production from iNOS reacts in conjunction with superoxide (also produced by activated immune cells). Immune cells congregate around the target cell and increase in number. NO is released from these cells and attacks copper and iron complexed proteins in the target cell. Copper and iron ions are released along with hydroxyl free radicals and molecular oxygen which leads to the destructive oxidative

injury to target cells (Thomas 2000).

The major function of NO from iNOS is the cytostatic and cytotoxic effects on invading microorganisms or tumour cells. The mechanism of NO induced cytotoxicity is in two categories. First is the inhibition of mitochondrial respiration. NO interacts and inhibits cytochrome c oxidase, the terminal acceptor of the electron transport chain (Cooper 2002). This is because these enzymes contain iron-sulphur centres in their catalytic sites. Second is the direct modulation of DNA synthesis due to inhibition of some enzymes *e.g.* ribonucleotide reductase. NO production by iNOS is essential for the defence mechanism of an organism, however NO produced by iNOS has been related to several pathological conditions. Unregulated NO synthesis becomes self-destructive and is known in autoimmune disease (Schmidt and Walter 1994); examples of such pathological conditions are diabetes (Corbett *et al* 1992), rheumatoid arthritis, Alzheimer's disease, impotence and schizophrenia (Lee *et al* 1999).

Poly (ADP-ribose) polymerase (PARP) is a DNA repair enzyme found within the nucleus of the cell which is activated by nicks and breaks in DNA. Breaks in DNA strands are caused by a number of different factors including environmental stimuli and free radical or oxidant attacks. NO produced from iNOS in response to pro-inflammatory conditions combines with superoxide to yield peroxynitrite. Peroxynitrite then induces single-strand breaks in DNA, which in turn activates PARP. PARP has been identified as a key final mediator of cellular injury in a variety of pathophysiological conditions such as diabetes, liver damage and ischemia-reperfusion (Szabo and Dawson 1998).

The understanding of the chemical and biological properties of NO is important to comprehend and appreciate the diversity of roles of NO and so lead to discovering its potential for disease therapy.

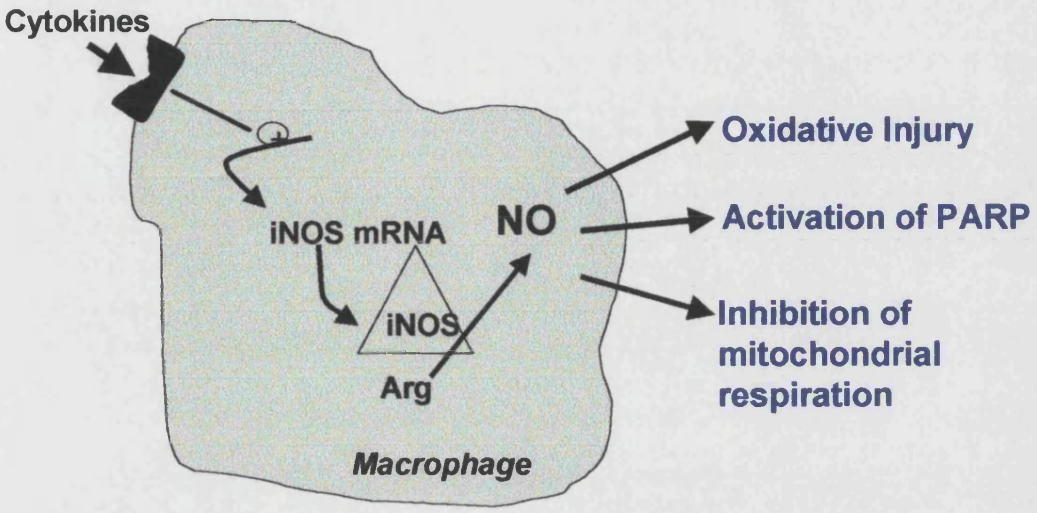


Figure 1.3: The pathway showing the production of NO by iNOS in a macrophage which has been stimulated by cytokines.

Structure of NOS

Each of the three isoforms contains four prosthetic binding domains for flavin-dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin and haem complex; iron protoporphyrin IX (haem) per monomer (Bredt *et al* 1991). All four of these cofactors are widely distributed in biological systems and help catalyse a number of oxidative and reductive reactions. However, the NOS isoforms are the only enzymes known to use all four simultaneously.

The active form of the NO synthase enzyme functions as a homodimer. This comprises of two oxygenase domains which are linked through interactions between haem domains and one reductase domain (Siddhanta *et al* 1996). The C-terminal reductase domain and the N-terminal oxygenase domain both fold independently of each other. The reductase domain is monomeric and contains one molecule each of bound FAD and FMN and also has a binding site for NADPH. The function of the reductase domain is to shuttle the five electrons from NADPH to the oxygenase domain. This electron transfer requires the binding of CaM. Studies have shown that only one haem unit per heterodimer is reduced and, in particular, the reduction will only occur when the arginine binding site and the reductase domain are on opposite subunits (Pfeiffer *et al* 1999). The oxygenase domain is homodimeric and contains a haem group and one binding site each for both the pteridine cofactor tetrahydrobiopterin and the substrate **1**. On the N-terminal side of the oxygenase domain, there is an extension which may play a role in positioning the enzyme correctly within the cell.

Between the two domains, there is a binding site for CaM. NOS is the only P-450 haem-containing enzyme in animals that has a dedicated reductase as an integral part of the protein; this suggests that the oxygenase-reductase interface is a crucial part of the function of the enzyme (Pfeiffer *et al* 1999). The CaM binding region acts as a hinge between the reductase and oxygenase domain. When Ca^{2+}/CaM is bound, the domains align and so the enzyme is active (Griffith and Stuehr 1995) (Figure 1.4). Binding of CaM accelerates both interdomain (from the reductase to the oxygenase domain) and intradomain (from NADPH through the reductase domain) electron transfer (Perry *et al* 2000).

The role of the tetrahydrobiopterin has been found to be essential in the reaction mechanism as the absence of the fully reduced form generates an excess of superoxide. One possible role for tetrahydrobiopterin is to act as a one-electron donor to the haem iron which is required for the cleavage of the oxygen–oxygen bond in the second step of the reaction mechanism.

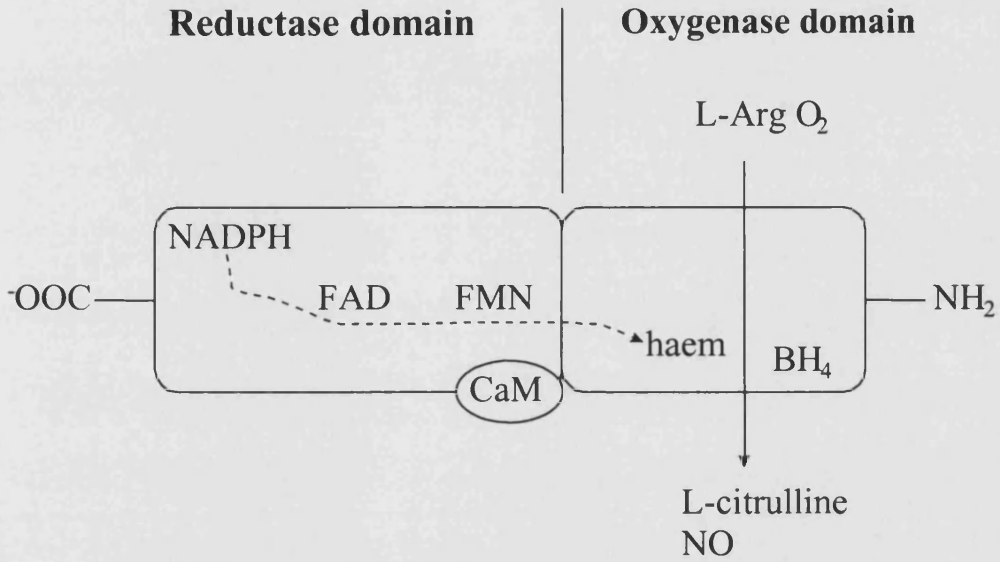


Figure 1.4: Domain structure of NOS. The dashed arrow represents the path of electron transfer (Pfeiffer *et al* 1999).

P450 Homology

Cytochrome P-450 enzymes contain a haem prosthetic group, an iron-sulphur cluster-containing protein and a flavoprotein reductase. These enzymes catalyse hydroxylation reactions. The flavoproteins and iron-sulphur proteins supply electrons from NADPH to cytochrome P-450 via a reductase which contains FAD and FMN (Poulos *et al* 1998). The P-450 part corresponds to the most intense absorption band of the carbon monoxide-liganded haem on the inhibited form. NOS appears to be the first self-sufficient mammalian P-450 enzyme (self-sufficient means that there is no need for a separate reductase). It is also unique in the fact that the reductase and haem domains are part of the same polypeptide (White and Marletta 1992). The flavin reductase is covalently linked to the haem domain via a linker that binds CaM (figure 1.5).

In both NOS and the homologous protein cytochrome P-450 reductase, the reductase domain is made up of nucleotide-binding molecules that bind NADPH, FAD and FMN. The only difference in their structure is an insert of approximately 45 amino acid residues within the FMN binding region. This insert is present in eNOS and nNOS but absent in iNOS (Pfeiffer *et al* 1999) (figure 1.5).

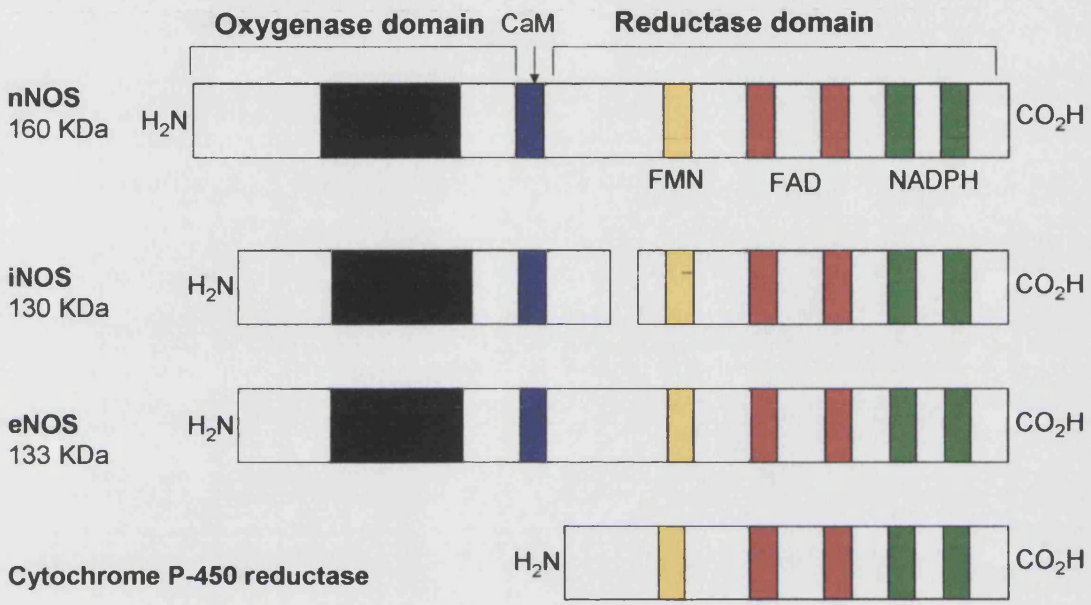


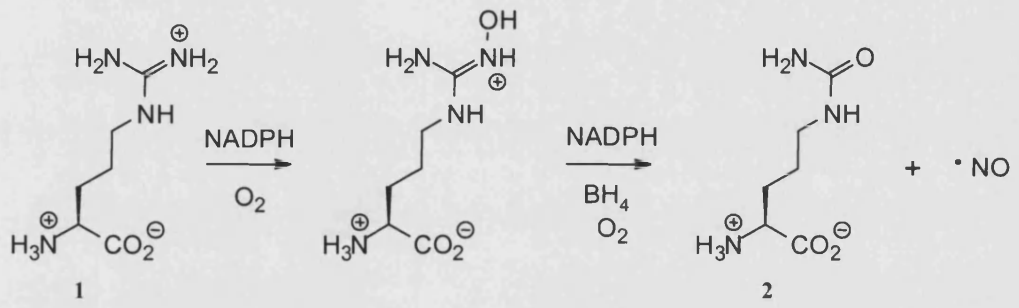
Figure 1.5: Structure of the NOS isoforms and cytochrome P-450 redcuctase cDNA. The gap in the iNOS sequence represents a 40 amino acid deletion (Ogden and Moore 1995).

It was first thought that NOS had significant homology to cytochrome P-450. Some of the chemistry that NOS displays is typical cytochrome P-450 chemistry. The P-450 reductase domain (C-terminal) the binding sequence for NADPH, FAD and FMN, is present in all isoforms. NOS also contains a cytochrome P-450 type iron protoporphyrin IX haem. This haem domain is most likely to be located near the N-terminus as it lacks the homology found in the C-terminal.

Common P-450 structural elements are missing from NOS, which suggests that it is most likely to be an example of convergent evolution and does not belong to the cytochrome P-450 superfamily (Marletta 1994a).

NOS Reaction Mechanism

NO is synthesised from the amino acid **1** by the enzyme nitric oxide synthase (NOS). Compound **1** undergoes a five-electron oxidation reaction to form L-citrulline (**2**) and NO (Marletta 1994b). Stable-isotope studies have provided the best evidence in understanding the mechanism of NO formation (Stuehr *et al* 1991). ^{15}N-labelled arginine showed that NO_2^- and NO_3^- (the breakdown products of NO) were derived from one of the guanidino nitrogens (Marletta 1993), and the ureido oxygen of **2** is derived from O_2 and not H_2O (Griffith and Stuehr 1995).



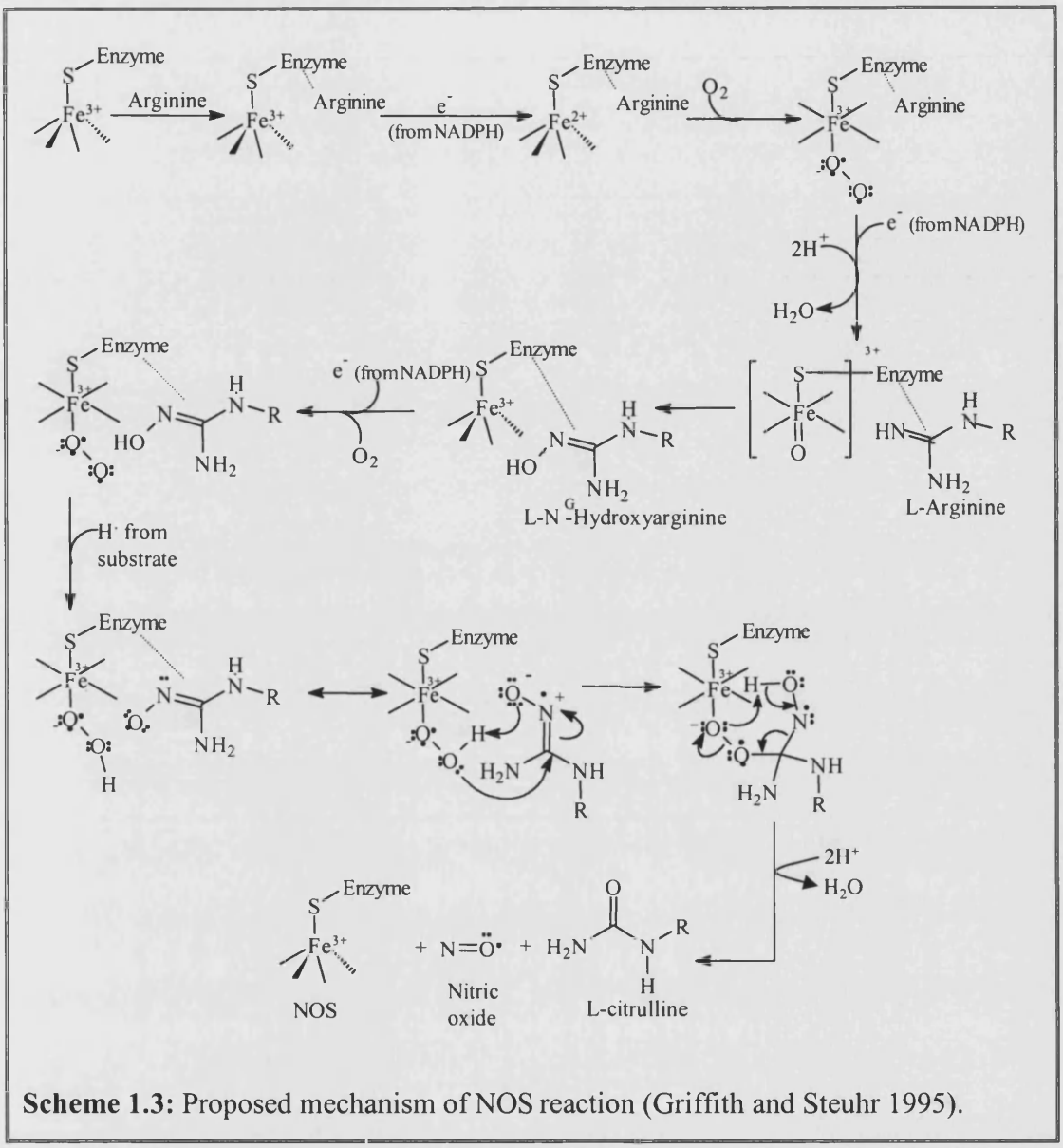
Scheme 1.2 NOS catalysed conversion of **1** to **2** and NO (Steuhr *et al* 1991). Tetrahydrobiopterin (BH_4).

The reaction is a five-electron oxidation of one of the guanidino nitrogen atoms of **1**. The first step is a two-electron oxidation to form the intermediate N^G-hydroxy-L-arginine (NOH-Arg). The second step involves an electron removal, oxygen insertion and a carbon-nitrogen bond scission to form **2** and NO in an overall three-electron oxidation. The electrons required for both steps in the reaction are provided by NADPH (scheme 1.2).

The reactions occur on the oxygenase domain of the enzyme. Compound **1** is bound near the haem co-factor (tetrahydropterin). The tetrahydrobiopterin is ligated to NOS through a sulphur atom on active site of a cysteine residue. The binding of **1** in the active site places both the oxidisable nitrogen and the guanidino carbon in close proximity to the haem iron (Babu and Griffith 1998a). The enzyme is in the ferric state and is firstly reduced to Fe^{2+} by an electron provided by NADPH via the flavins. The ferrous form then binds oxygen to give a stable complex. It is the

dissociation of this species that is likely to be the source of superoxide ion formation. Simultaneous addition of an electron delivered from NADPH *via* the flavins FAD and FMN and a proton from the BH_4 complex reduces the bound superoxide to hydroperoxide. The breaking of the oxygen-oxygen bond results in water being released and the perferryl haem species is formed (Alderton *et al* 2001). The perferryl haem species is a potent hydroxylating agent and so rapid hydroxylation occurs in which **1** is oxygenated to the intermediate NOH-Arg and the ferric haem is regenerated (Babu and Griffith 1998a).

The second stage in the reaction is unique to NOS. The original haem cofactor receives an electron from NADPH and binds a second molecule of oxygen. This ferric-oxy haem complex may act as an oxidant, by removing an electron from the bound NOH-Arg as an $H^{\cdot}$ radical. The peroxy-iron species carries out a nucleophilic attack at the cation radical to generate a tetrahedral intermediate. This rearranges to give **2**, NO, water and ferric haem (Korth *et al* 1994) (scheme 1.3).



Scheme 1.3: Proposed mechanism of NOS reaction (Griffith and Steuhr 1995).

NOS Domain Structure

Little has been known about the structure of the NOS active site until recently. By understanding the structure of each domain, more information can be gained about NOS activity and the mechanism of action both of which are important in developing new NOS inhibitors.

Since the publication of NOS crystal structures, more information has been available about binding of inhibitors to these active sites and, indeed, where they bind. Although, as expected there is much homology between the active sites in the three isozymes, there are subtle differences, particularly in the distal pockets.

The haem and tetrahydrobiopterin cofactors together with the oxygenase domain form the active site of the enzyme (Perry *et al* 2000). The oxygenase domain of iNOS appears like a baseball catcher's mitt with the haem group in the palm of the mitt. Dimerisation creates a 30 Å deep funnel-shaped active centre. The distal pocket of NOS differs from other haem-based oxygenases in the fact that it is mainly comprised of β-sheets, whereas other haem-based oxygenases are helical (Crane *et al* 1998). Each of the isozymes have slightly different structures in these distal pockets, which influences the catalytic and ligand binding properties. At the centre of the dimer interface, there is a zinc tetrathiolate which aids in dimerisation stability and also in the formation of the pterin binding site (Li *et al* 2001). The binding of tetrahydrobiopterin, haem and **1** all promote and stabilize the active dimeric forms of all three isoforms of NOS.

Many of the arginine analogue inhibitors bind in the substrate binding site. Imidazoles act in a different way, by preventing the formation of **2**. The unbonded electron pair on position three of the imidazole ring acts as a sixth ligand to the haem iron and prevents oxygen binding (Wolff *et al* 1993). Crystallographic studies allow information to be gained about where the inhibitor is binding and also the hydrophobic interactions involved.

Therapeutic potential of NOS inhibitors

The development of NOS inhibitors has immense potential in the medical world, as NO is linked to many disease states. NO produced from iNOS has been linked to autoimmune disease states such as arthritis (Evans and Stefanovic-Racic 1997) and diabetes (McDaniel *et al* 1996). nNOS-derived NO is linked with diseases related to the brain, such as Parkinson's disease (Gatto *et al* 2000), Huntington's disease (Bryk and Wolff 1999) and migraine headaches (Babu and Griffith 1998a). NO is not only involved in disease states but is also essential for human health; for example, eNOS is tightly regulated and plays a critical role in the maintenance of blood pressure (Babu and Griffith 1998a) and so the design and synthesis of highly selective NOS inhibitors is paramount. Failure to achieve selectivity can lead to many systemic side effects and so therapeutic applications of the inhibitor become limited. Inhibition of eNOS is not ideal as it may lead to a large increase in blood pressure with many profound side effects and so selective eNOS inhibitors do not appear to have such a great potential in drug development.

Several inhibitors have been tested *in vivo* and have produced encouraging results, e.g. the iNOS selective inhibitor 1400W has been shown to reduce the rate of solid tumour growth in mice (Thomsen *et al* 1997). The administration of other NOS inhibitors has shown significant effects in other disease states such as arthritis and septic shock. L-NAME was administered to rats induced with adjuvant arthritis. Adjuvant arthritis in rats is an experimental immunopathy which involves a T-lymphocyte-mediated delayed hypersensitivity reaction. Rats receiving the L-NAME therapy showed a marked decline in their T-lymphocyte response (Ialenti *et al* 1993). The cardiovascular collapse that is associated with septic shock is largely due to endotoxin. L-NMMA has been shown to have a rapid and strong anti-hypotensive effect in endotoxin-induced dogs (Kilbourn *et al* 1990).

NOS inhibitors can generally be divided into two categories, amino-acid derivatives and non-amino-acid derivatives. These will be discussed in turn.

Amino-Acid derivatives

L-Arginine analogues

The arginine analogues include the first NOS inhibitors synthesised. Studies have shown that the L- configuration is a requirement for the inhibitor as D-arginine is not a substrate and does not inhibit the NOS reaction (Narayanan and Griffith 1994).

The arginine analogues include N^G-monomethyl-L-arginine (L-NMMA (**4**)), N^{ε}-(iminoethyl)-L-lysine (L-NIL (**5**)) and its lower homologue N^{δ}-(iminoethyl)-L-ornithine (L-NIO (**6**)), N^G-nitro-L-arginine (L-NNA (**7**)) and its methyl ester (L-NAME (**3**)). The mechanism of NOS inhibition by these compounds varies, but they all bind to the oxygenase domain of NOS and interact with the guanidinium region of the arginine binding site (Moore *et al* 1994). Compounds **3**, **4** and **6** are reversible competitive inhibitors, which show potency but no particular selectivity for any type of NOS (Ogden and Moore 1995), the effects of which are reversed by the addition of arginine. IC_{50} values for arginine analogues are given in table 1.2. Lead target inhibitors should display at least 100-fold isoform selectivity.

The arginine analogues differ in their influences on electron transfer and in the reduction of the haem iron in the NOS enzyme. Compound **4** binds with its unsubstituted guanidinium nitrogen in the distal, non-reactive guanidinium nitrogen pocket (Babu *et al* 1999), and has been found to be a mechanism-based inhibitor. It is first hydroxylated to N^G-hydroxy-N^G-methyl-L-arginine which is then demethylated to intermediate in the NOS reaction N^G-hydroxy-L-arginine and finally metabolised to **2** and NO (Klatt *et al* 1994).

Table 1.2 shows that compound **7** displays selectivity for the cNOS isoform. This is due to the higher affinity of **7** for the nNOS isoform (Furfine *et al* 1993). The methyl ester of compound **7** (**3**) is a much less potent NOS inhibitor (IC_{50} 70 μM brain NOS). The inhibitor only inhibits cNOS in pharmacological experiments with animals or tissues following its hydrolysis to the free acid **7** (Pfeiffer *et al* 1996). Compound **3** acts by inhibiting NADPH consumption by interrupting electron flow immediately prior to reduction of the haem iron (Southan and Szabo 1996). The electron flow is blocked by inhibiting haem iron reduction which involves a lowering

of the haem iron reduction potential (Abu-Soud *et al* 1994).

Compound **5** shows selectivity for the iNOS isoform while its lower homologue **6** shows potency but no isoform selectivity. Compound **6** binds to the guanidium region of the arginine binding site and is thought to be oxidised in a similar way to **1** (Fast *et al* 1999).

Compounds **4**, **6** and **3** have been shown to increase blood pressure and so inhibit NO production from endothelial cells both *in vivo* and *in vitro* (Rees *et al* 1990). Unfortunately, due to the modest selectivity displayed by these compounds, many undesirable side effects have occurred in animal studies, indicating the need for more selective NOS inhibitors (Babu and Griffith 1998b).

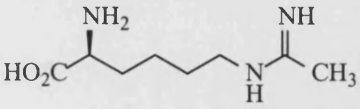
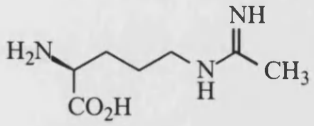
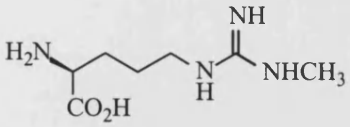
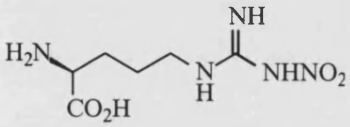
	IC_{50} (μM)	
	cNOS (rat)	**iNOS (mouse)**
 **L-NIL (5)**	92	3.3
 **L-NIO (6)**	3.9	2.2
 **L-NMMA (4)**	8.3	18
 **L-NNA (7)**	0.5	20

Table 1.2: IC_{50} Values for arginine analogues. Values taken from Moore *et al* 1994

The most potent and selective of the N^{ω}- substituted arginine analogues is N^{ω}-propyl-L-arginine. It was found to be a competitive inhibitor of all three isoforms. The K_i values show that there is substantial degree of selectivity for the nNOS isoform (from bovine brain). Selectivity for nNOS/iNOS is the ratio of the inverse of the K_i values. N^{ω}-propyl-L-arginine shows a potency of inhibition for nNOS which is 3158 times that for iNOS (mouse murine recombination (Hevel *et al* 1991)) and 149 times that for eNOS (bovine endothelial recombinant (Martasek *et al* 1996)). The propyl chain length (compared to methyl, ethyl, allyl and propargyl) gives rise to the optimum size and geometry for nNOS selective inhibition for this class of compounds (Zhang *et al* 1997). This suggests that the binding region for the guanidinium nitrogen which is located near the haem iron can accommodate substituents extending about 4-5 Å from the guanidinium carbon (Babu *et al* 1999).

L-Citrulline analogues

Thiocitrulline

Compound **2** (Scheme 1.2), the amino acid product of the NOS reaction is not a useful inhibitor. However, when the ureido oxygen is replaced by sulphur, this gives rise to potent inhibitors. Thiocitrulline (**8**) competitively inhibits NOS showing a degree of selectivity for the iNOS isoform (table 1.3). Compound **8** acts by decreasing the electron flux through NOS and NADPH oxidase activity of NOS by lowering the reduction potential of the haem iron (Ware and King 2000).

X-ray crystallographic studies of iNOS oxygenase domain show that **8** binds with its sulphur atom positioned directly above the haem iron. Compound **8** binds to the active site in a similar conformation as does L-arginine. It binds in the distal pocket (which is adjacent to the haem) by interacting directly with Glu371 (Crane *et al* 1997). The inhibitor is held in place by hydrogen bonds being formed between the carboxyl oxygen atom of Glu371 and the sulphur atom in the thiourea group of **8** (Ware and King 2000). Frey *et al* 1994 have shown that the interaction between the haem iron and homothiocitrulline (**9**) are not as strong as in **8**. This is because the sulphur atom is further away from the haem iron and so the ability to alter the reduction potential of the haem iron is affected. However, the percentage inhibition results indicate that **9** is a reasonable NOS inhibitor and so other binding interactions

must be occurring between NOS and the thioureido unit (Frey *et al* 1994).

S-methylthiocitrulline (**8a**) and *S*-ethylthiocitrulline (**8b**) are potent reversible slow-binding inhibitors of NOS (Furfine *et al* 1994). They bind to the enzyme active site in the same way as **8** and **9**. Compound **8a** displays a degree of selectivity for nNOS (table 1.3). The alkylation of the sulphur atom in **8** increases the strength of the interaction with the enzyme this is evident from the IC_{50} values given in table 1.3. The alkyl functionality attached to the sulphur atom increases the potency of the inhibitor; this is also seen in isothiourea compounds, as synthesised by Garvey *et al* 1994. The alkyl group may give rise to an increase in potency by binding into a small hydrophobic pocket region within the arginine binding site.

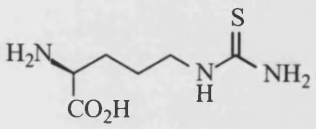
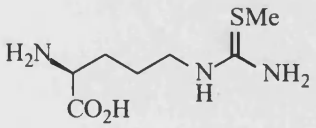
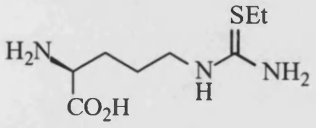
	IC_{50} (μM)	
	nNOS (rat)	**iNOS (rat)**
 **Thiocitrulline (8)**	4.6[a]	1.7[a]
 ***S*-methyl thiocitrulline (8a)**	1.05[b]	2.2[b]
 ***S*-ethyl thiocitrulline (8b)**	1.56[b]	1.56[b]

Table 1.3: IC_{50} Values for citrulline analogues. a Ulhaq *et al* 1999, b Narayanan *et al* 1995

Non-amino-acid inhibitors

Non-amino-acid inhibitors are another class of NOS inhibitor. These include isothioureas, 1400W (**10**), aminoguanidine (**11**), 7-nitroindazole (7NI (**12**)) and imidazoles. Many of the non-amino-acid inhibitors show isoform-selectivity, particularly for iNOS and so important structural features can be determined in order to design and synthesise potent selective inhibitors and also gain information about binding to the NOS active site.

Aminoguanidine

Aminoguanidine (**11**) is one of the first non-amino-acid inhibitors to be identified and inhibits NOS in a time-dependent manner (Wolff and Lubeskie 1995). Other aminoguanidine analogues have been studied, but **11** displays the greatest selectivity for iNOS (Babu and Giffith 1998a) (Table 1.4).

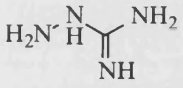
	IC_{50} (μM)		
	iNOS	**nNOS**	**eNOS**
 **Aminoguanidine (11)**	5.0	41.0	255

Table 1.4: IC_{50} Values for aminoguanidine. Values taken from Wolff *et al* 1998.

Due to its low acute toxicity, **11** has been used in clinical therapeutics including various experimental models of inflammation and shock (Southan and Szabo 1996). Compound **11** has also been shown to inhibit vascular changes in diabetic rats which cause increase blood flow (Corbett *et al* 1992).

Isothioureas

The isothioureas are a group of inhibitors which have been shown to be competitive with arginine and therefore are known to bind to the arginine binding site on the haem-domain. Several of the inhibitors within this group are selective for iNOS. S-ethylisothiourea (EIT (**13**)) and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT (**13a**)) inhibited murine iNOS *in vitro* with IC_{50} results of 13 and 4 nM,

respectively (Nakane *et al* 1995).

S-alkylisothioureas have been shown to be potent NOS inhibitors. In particular, **13** shows potency and selectivity for iNOS. The potency is believed to be directly related to the steric bulk of the S-alkyl substituent. Shearer *et al* (1997) showed that optimal potency is observed for S-ethylisothiourea. The introduction of the phenyl ring in S-ethyl-N-phenylisothiourea (**14**) led to nNOS selectivity. The type of substituents bonded to the aromatic ring as well as the position play an important role in the selectivity of the inhibitor, with para electron-withdrawing groups giving rise to the most selective inhibitors (Shearer *et al* 1997).

Garvey *et al* have developed a series of bisisothioureas, which have produced encouraging results *in vitro*. In particular S,*S*'-(1.3-phenylenebis-(1,2-ethanediyl))bisisothiourea (PBITU (**14a**)) and S,*S*'-(1.4-phenylenebis-(1,2-ethanediyl))bisisothiourea (**14b**) showed potency and selectivity for human iNOS giving K_i values of 0.047 μM and 0.0074 μM respectively. However their poor cellular penetration and toxicity may limit their therapeutic use (Garvey *et al* 1994).

1400W

N-(3-(Aminomethyl)benzyl)acetamidine, known as 1400W, (**10**) is the most selective inhibitor of iNOS to date. Compound **10** was shown to be 5000 and 200-fold selective for human iNOS against eNOS and nNOS respectively (Garvey *et al* 1997). (As mentioned earlier selectivity can be defined as the ratio of K_i eNOS or K_i nNOS to K_i iNOS). The selectivity of **10** towards iNOS may be partly attributed to the higher turnover of iNOS compared to nNOS and eNOS (Li *et al* 2001) (table 1.5).

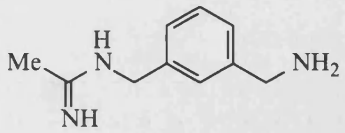
	IC_{50} μM		
	iNOS	**nNOS**	**eNOS**
 **1400W (10)**	0.23	7.3	1000

Table 1.5: IC_{50} values for 1400W (Alderton *et al* 2001)

Compound **10** binds competitively with **1**. Both **6** and **10** share the same amidine group which is structurally similar to guanidine and so the amidine binds in the guanidine-binding pocket of the substrate site, H-bonding with Glu363. Inactivation of iNOS is believed to occur after the oxidation step but before hydroxylation (scheme 1.3). Compound **10** is unable to donate a proton to the ferric-hydroperoxy intermediate which leads to an excess production of peroxide and haem destruction (Li *et al* 2001).

Compound **10** is a time-dependent inhibitor of iNOS and most of the selectivity of iNOS was seen when the interactions develop slowly. When the enzyme was pre-incubated with **10**, an optimum pre-incubation time of ten minutes was obtained (Garvey *et al* 1997). The progressive nature of inhibition for iNOS (not eNOS or nNOS) accounts for the underestimated IC_{50} value for iNOS given in table 1.5. This also explains why the selectivity seen in table 1.5 is much lower than that observed by Garvey *et al* 1997.

The removal of the methylene unit from **10** to give N-(3-(aminomethyl)phenyl)acetamidine (**15**) reversed the selectivity of the compound to give rise to a nNOS selective inhibitor, which displays no time dependence kinetics but binds reversibly (Collins *et al* 1998). As seen in the isothiourea group, the position of the substituents on the aromatic ring plays a role in the selectivity of the inhibitor. This was also demonstrated by Collins *et al* as shifting the aminomethyl functionality from the meta to the para position resulted in a reduction in nNOS and

eNOS inhibition but not in iNOS inhibition (Collins *et al* 1998).

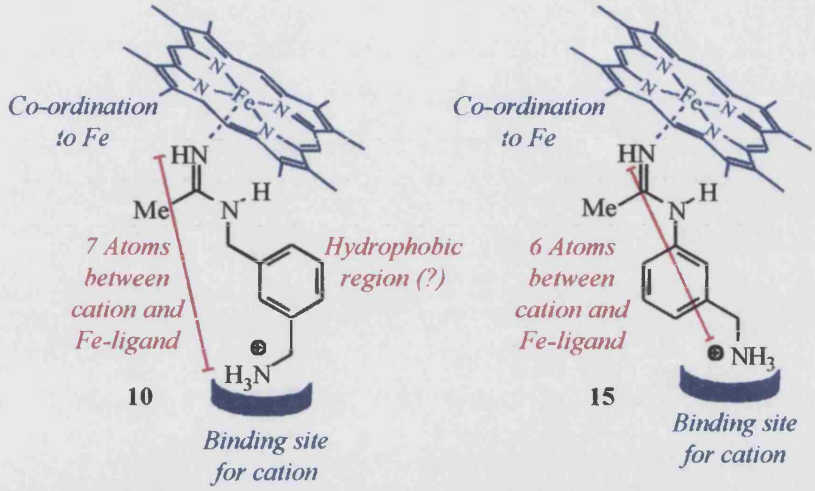


Figure 1.6: Proposed binding of the iNOS selective inhibitor **10** and the nNOS selective inhibitor **15**, illustrating the co-ordination of the amidine head group to the haem and the bridge length of the compound which may play an important role in the selectivity of the inhibitor.

Compound **10** was shown to be highly selective both *in vitro* and *in vivo*. It shows a similar toxicity profile to another selective iNOS inhibitor **14a** and so use of this drug in human therapeutics is unsafe, but can be used effectively in animal models. However, dose-limiting toxicity has been observed for this compound (Thomsen *et al* 1997). Compound **10** has been shown to reduce the rate of growth in solid tumours in mice.

Imidazoles

The imidazoles are a group of non-selective inhibitors. Imidazoles have been shown to be non-competitive inhibitors of iNOS and nNOS while being competitive inhibitors of cNOS (Aktinson and King 1999).

The consensus for the mode of action for the imidazoles is that they block the formation of the reaction product (**2**). The unbonded electron pair on position three of the imidazole ring acts as a sixth ligand to the haem iron. The binding of the imidazole prevents oxygen binding and therefore prevents the formation of citrulline

(Wolff *et al* 1993).

The selectivity and potency of the imidazole inhibitors is closely linked to the amino acid chain length attached to the head group. Atkinson and King (1999) and also Lee *et al* (1999) found that the longer chain lengths decrease the potency of the inhibitor. Substitution of the imidazole head group also affects the potency of this class of inhibitors. Substitution at positions two and four on the ring have been shown to diminish the potency. This may be due to steric hindrance (Ulhaq *et al* 1998), whereas substitution at position one in particular with an alkyl or aromatic group is favoured (Salerno *et al* 2002).

IC_{50} Values for various imidazole NOS inhibitors are given in table 1.6.

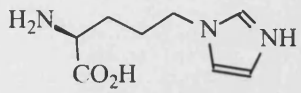
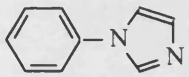
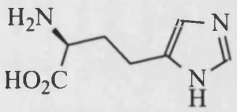
	IC_{50} μM		
	iNOS	**nNOS**	**cNOS**
 **(S)-2-Amino-5-(imidazol-1-yl)pentanoic acid (16)**	32[a]	19[a]	13[a]
 **Imidazole (16a)**	59[b]	175[b]	189[b]
 **Phenyl imidazole (16b)**	33[b]	429[b]	11[b]
 **(*S*)-2-Amino-4-[1(3)*H*-imidazol-4-yl]butanoic acid (16c)**	950[c]	170[c]	500[c]

Table 1.6: IC_{50} values for imidazoles. a Ulhaq *et al* 1998 rat isoforms used, b Chabin *et al* 1996 human isoforms used, c Lee *et al* 1999 mouse iNOS, bovine eNOS and rat nNOS used (Ki μM values).

Indazoles

Compound **12** is the most studied nNOS-selective inhibitors (Southan and Szabo 1996). However, the selectivity seen in **12** for nNOS remains low *in vitro* (Schumann *et al* 2001). Indoles are believed to bind to the NOS haem iron in the same way as the imidazoles by binding to the haem iron instead of oxygen and preventing the formation of **2** (Wolff and Gribin 1994).

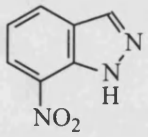
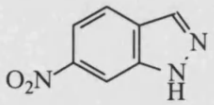
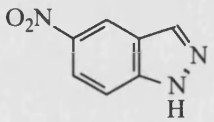
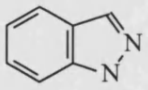
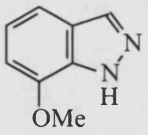
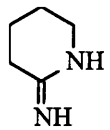
	IC_{50} μM (nNOS)
 **7NI (12)**	2.5[a]
 **6NI (12a)**	40[a]
 **5NI (12b)**	115[a]
 **Indazole (12c)**	230[a]
 **7MI (12d)**	6.3[b]

Table 1.7: IC_{50} values for Indoles. a bovine brain NOS Wolff and Gribin 1994, b rat cerebellar NOS Schumann *et al* 2001.

As table 1.7 shows, the nitro group at position seven gives rise to the optimum potency for this particular series. By moving the nitro group to either position six or five, the potency of the inhibitor decreases. The nitro group on an aromatic ring is strongly electron-withdrawing and decreases the electron density of the aromatic ring. Also, the nitro group contains a semipolar bond that leaves the nitrogen atom with a positive charge and the oxygen atoms with delocalised negative charges. It is unclear whether the potency seen in **12** is due to the change in electron charge distribution of the aromatic ring or if the positively charged nitrogen atom interacts in some way with NOS. However the potency displayed by 7-methoxyindazole (7MI **12d**) contradicts the theory that potency displayed by **12** is due to the electron withdrawing effect of the nitro group at position seven (Schumann *et al* 2001).

Other non-amino acid inhibitors

Another class of potent NOS inhibitors include the cyclic amidines. 2-Iminopiperidine (**16d**) is one of the most potent inhibitors within this series; IC_{50} 1.4, 4.7, 1.1 μM against human iNOS, cNOS, nNOS respectively. The potency of the inhibitor depends greatly on the position and functionality of the groups substituted around the ring. The effect of methylation at the different ring positions has been investigated and positions four and six increased the potency of the inhibitor relative to **16d** whereas positions three and five showed a decline in NOS inhibition (Webber *et al* 1998). The size of the piperidine ring also affects the potency of the inhibitor with seven membered rings giving rise to similar potency to that seen in six membered rings values whereas five, eight and nine membered rings decreased the potency of the inhibitor (Moore *et al* 1996).



2-iminopiperidine (16d)

Other non amino acid inhibitors include porphyrins (Wolff *et al* 1996), pyrazole N-carboxamidines (Southan *et al* 1997 and Lee *et al* 2000). All of these three classes of NOS inhibitors are potent but not particularly selective for any particular isoform.

Tumour Biology

Normal cells reproduce when instructed to do so by other cells, this process is known as the cell cycle. Cancer cells arise when alterations occur in the control mechanisms of the cell cycle causing cancer cells to undergo their own reproduction.

The cell cycle is a complex assembly of stimulatory and inhibitory pathways. There are four stages to the cycle; G_1 pre-replication stage in which the cell enlarges and prepares to copy DNA. G_0 is the resting phase, S is the synthesis phase and M is mitosis (cell division). The critical stage in the cycle is the restriction point (R), which occurs late in the G_1 phase. At this point, the cell decides whether or not to commit itself to completing the cycle (Weinberg 1996) (figure 1.7).

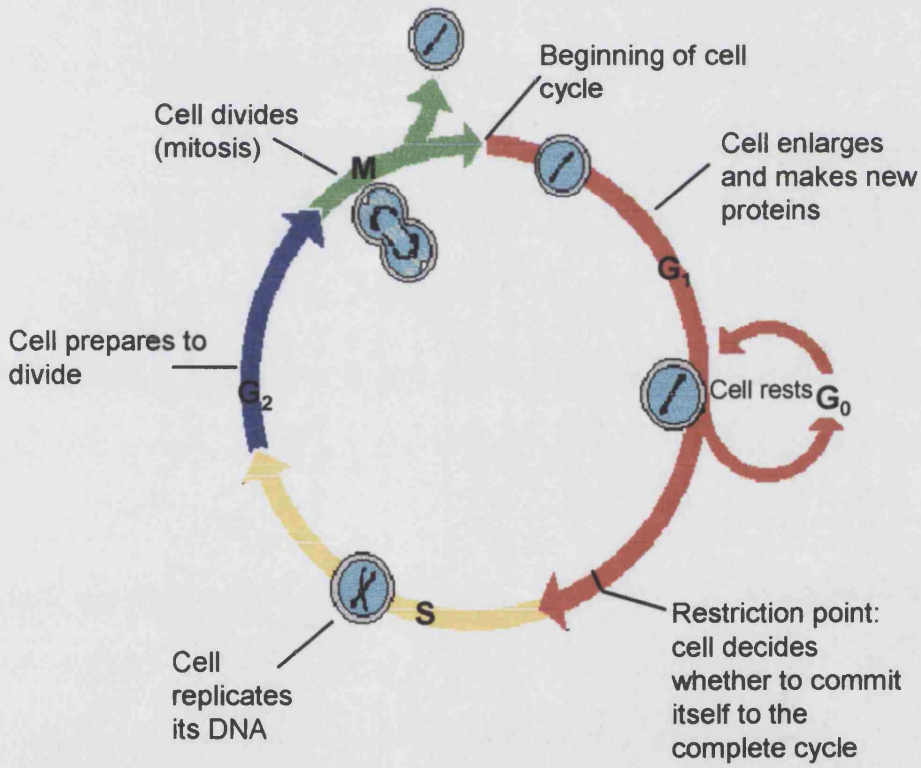


Figure 1.7: The four stages of the cell cycle; G_1 pre-replication stage, G_0 is the resting phase, S synthesis and M is mitosis (cell division).

There are two gene classes that control the life cycle of the cell; proto-oncogenes which encourage cell growth, while tumour suppressor genes inhibit it. For a

cancerous tumour to develop, mutations must occur in at least six of the controlling genes. Mutated proto-oncogenes (oncogenes) cause excessive cell multiplication while mutations in tumour-suppressor genes result in a loss of a functional tumour suppressor protein and so deprive the cell of brakes that prevent inappropriate growth.

In order for a cancer cell to metastasise successfully, they must detach from their original location and invade the blood or lymphatic system. The survival of the tumour relies on the cancer cells establishing their own blood supply (angiogenesis) (Weinberg 1996).

Cancer Therapy

The current main therapies available for internal tumours are; surgery, chemotherapy and radiotherapy. These therapies often have such harmful side effects and so their administration often compromises the benefits of treatment. The need for safe and effective therapies that act only on cancer cells is paramount.

Surgery

Surgery is the earliest established therapy for tumours and is still the most widely used. The removal of the tumour and eradicating the cancer from the body is often the most effective way. However there are several pitfalls from this type of treatment. The physician may be able to remove the tumour with the scalpel but often microscopic parts of the tumour are left behind and so the tumour is able to metastasise again. Surgery often involves the removal of healthy tissue to ensure that the tumour has been fully excised; however; this can leave the patient with severe damage and discomfort (Hellman and Vokes 1996).

Chemotherapy

Chemotherapeutic drugs act by interfering with the cells DNA and so preventing the tumour cells from replicating. Some of these drugs act directly on tumour cells whereas others must be activated by metabolic processes, either in the tumour cells or in organs (Franks and Teich 1995). Chemotherapeutic drugs can be divided into three groups according to their site of action. The first group are only active on dividing cells. The second group are active on dividing cells and affect a particular phase of the cell cycle; an example is methotrexate which is active in the S phase of the cell cycle. The final group are those drugs which affect all or most of the phases of cell division; an example is nitrosourea (Franks and Teich 1995).

Chemotherapy affects both tumour cells and normal cells; the feasibility of chemotherapy is based on the fact that normal tissue cells may recover at a faster rate than tumour cells. During the 1940s when chemotherapeutic drugs were first developed their effectiveness often proved inadequate when administered alone. By the 1960s physicians discovered that the success rate increased when several drugs were given at the same time. Leukemias and testicular cancer are two examples that

are now successfully treated with combination therapy (Hellman and Vokes 1996).

Besides killing normal cell tissue chemotherapeutic drugs often cause many serious side effects such as damage to the growing cells of the bone marrow causing anaemia due to the patient being unable to produce adequate number of red blood cells, white blood cells and platelets. Other side effects include diarrhoea, nausea, and vomiting and hair loss. Probably the major problem with the failure of chemotherapy is drug resistance by the cancer cells. Tumours can develop a resistance to multiple drugs after the administration of one drug (Franks and Teich 1995).

Radiotherapy

Radiation therapy involves the use of powerful X-rays or gamma rays to irradiate the tumour. Radiation treatments act in two ways; either by inflicting sufficient genetic damage to kill the cells directly or by inducing apoptosis. Radiation enables the microscopic amounts of tumour that surgery may leave behind to be destroyed; also healthy tissue is able to recover more easily. One of the factors that limits the effectiveness of radiotherapy is the presence of radioresistant hypoxic tumour cells (Wink *et al* 1998). These cells have PO_2 levels of < 10 mm Hg and so much effort has been made to exploit these regions of tumour cells to develop drugs that are effective hypoxic cell radiosensitizers (Sutherland *et al* 1988) (figure 1.8).

Anti-angiogenic therapy

Angiogenic therapy does not aim to destroy the tumour but exploits the fact that tumours require their own blood supply to survive. Angiotherapeutic drugs act by limiting the blood supply and so shrinking the tumour and preventing it growing. The main advantage of this type of therapy is the fact that the drugs are specially designed to be specific for tumour cells and so prevent healthy cells being destroyed. This type of therapy is relatively new and as yet no drugs have been approved for use in patients but many are now in clinical trials (Folkman 1996).

The type of treatment a patient receives depends on the tumour and the stage of progression. Many physicians combine the use of surgery, radiotherapy and chemotherapy to provide the most effect way to eradicate the tumour.

NOS and Cancer Therapy

NO is generated by a variety of cell types including several tumour cell lines and solid human tumours (Edwards *et al* 1996). The production of NO is carried out either by the tumour cells themselves, by the endothelial cells in the tumour vasculature or by the macrophages and stromal cells within the tumour (Lala and Orucevic 1998). The effects of NO production by tumour cells include; tumour cell growth, differentiation, metastatic capability, chemosensitivity, radiosensitivity and tumour-induced immunosupression (Edwards *et al* 1996).

NO may play a dual role within the tumour cells. High concentration of NO may participate in antitumour events (Edwards *et al* 1996) such as the induction of tumour apoptosis and the inhibition of tumour growth. However, when NO concentrations are low tumour growth and angiogenesis may be stimulated (Jenkins *et al* 1995). Another factor affecting the dual role of NO within tumour cells is NO sensitivity. Different tumour types may have different sensitivities to NO (Buttery *et al* 1993).

The role(s) that NO plays within the tumour remain unclear and conflicting. However, it is believed that the function of NO within tumour cells depends on the stage and type of tumour and also the genetic make-up within the cell (Doi *et al* 1996). The activity of NO has been positively correlated to the degree of malignancy in human ovarian (Lala and Orucevic 1998), uterine, breast (Thomsen *et al* 1995, Jenkins *et al* 1995) and gynaecological tumours (Thomsen *et al* 1994). Tumours of the central nervous system have been found to have high expression of cNOS and nNOS (Cobbs *et al* 1995).

The administration of the non-competitive arginine analogue NOS inhibitors **3**, **4** and **7** have been shown to decrease the blood flow to lung metastases (Edwards *et al* 1996), murine adenocarcinoma (Andrade *et al* 1992) and rat carcinoma (Tozer *et al* 1997). However, there is conflicting evidence that these effects are reversed by the addition of L-arginine (Meyer *et al* 1995). The growth rate of tumours has been shown to decrease with the administration of **3** and **4** in C3L5 tumour-bearing mice (Lala and Orucevic 1998). These results are very encouraging but it is important to remember that these inhibitors are non-selective and so caution must be exercised

when interpreting this data.

The administration of the iNOS selective inhibitor **10** has been shown to reduce the rate of solid tumour growth in mice; however, the murine colon adenocarcinoma showed no reduction in growth rate after treatment with **10** (Thomsen *et al* 1997).

The mechanism of how NO affects tumour proliferation is unclear but there are several possibilities. i) The breakdown of the extracellular matrix is believed to be an important process in the invasion and metastasis of solid tumours. The enzymes responsible for the breakdown of the matrix are known as metalloproteinases. One mechanism for the role of NO in tumours is through the activation of metalloproteinases (Thomsen *et al* 1997). ii) the direct stimulation of tumour cell proliferation, iii) promotion of tumour cell invasiveness iv) promotion of tumour angiogenesis v) promotion of microcirculation in tumour vasculature and vi) the suppression of host cell defence (Lala and Orucevic 1998).

Although more detailed understanding of the mechanism of action is required, the potential for selective iNOS inhibitors in the treatment of cancer therapy is immense.

Many solid tumours have regions of low oxygen tension, which are known as hypoxia (figure 1.8). These areas are thought to be a result of poor and disorganised blood supply. These cells are generally radio- and chemoresistant and so can cause a relapse of the cancer after such therapy. Severe hypoxia is unique to tumours and therefore has great potential to be exploited in cancer drug development (Chinje and Stratford 1997). Hypoxia is known to provide a stimulus for angiogenesis and one such pathway for this action may be through the activation of the iNOS promoter via the hypoxia response element (HRE) (Lala and Orucevic 1998). Inhibition of NOS has been shown to decrease tumour size, which correlates with an increase in the level of tumour hypoxia sufficient enough to enhance the efficiency of bioreductively activated drugs (Wood *et al* 1993).

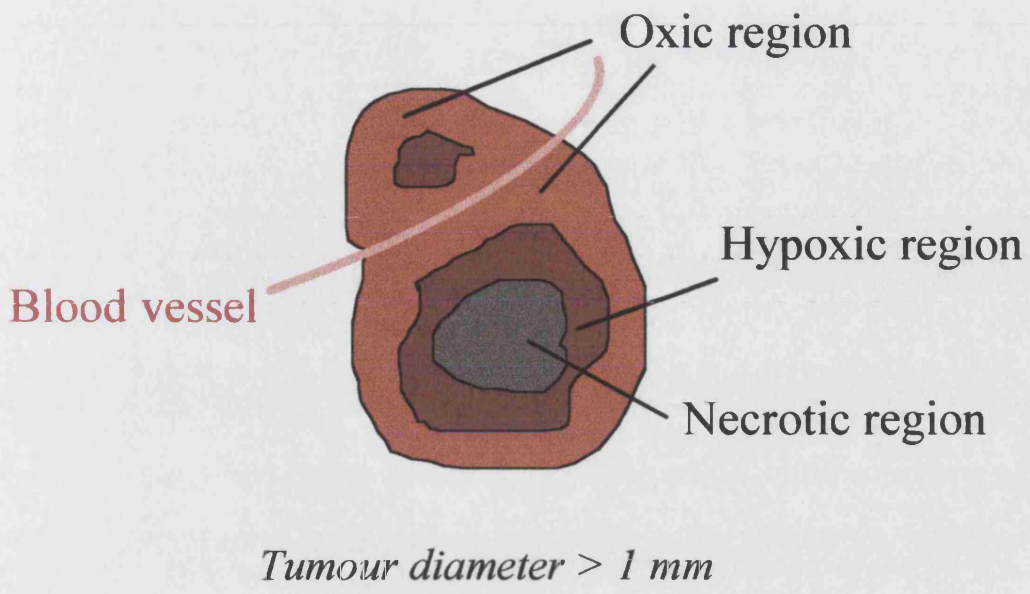


Figure 1.8: Diagram to show the hypoxic regions within a solid tumour.

As mentioned earlier NO inhibition reduces blood flow to the tumour. The levels of hypoxia have been shown to increase in those tumours which have received NOS inhibition. This evidence provides an approach to developing selective NOS inhibitors which will reduce blood supply to the tumour and cause vascular shut-down of the tumour and also potentiate the use of bioreductive anti-cancer drugs.

This is the main objective of the project and will be discussed in detail in chapter two.

Aims, Objectives and Research Proposal

Aims

The potential for the use of selective NO inhibitors in diminishing tumour blood flow is immense. The arginine analogues, **4**, **3** and **7** are potent but non-selective inhibitors and so are likely to cause systemic side effects. The most potent NOS inhibitor to date is **10**, which is selective for the iNOS isoform (Garvey *et al* 1997). It remains uncertain whether the selectivity shown by this compound is partly due to the high turnover rate of iNOS when compared with eNOS and nNOS (Li *et al* 2001). Also **10** is highly toxic, so the need for potent non-toxic NOS inhibitors still exists.

The aim of this research project was to synthesise inhibitors of the inducible and constitutive isoforms of NOS, which would be highly selective and so eliminate side effects associated with non-selective inhibitors. Selective inhibition in tumours should achieve collapse of the tumour and shutdown the tumour vasculature. Such selective inhibitors may alone cause an anti-tumour event. However it could also increase tumour hypoxia and potentiate the cytotoxicity of bioreductively activated drugs.

Objectives

The objectives of this research project are as follows;

i) Modify the structures of N^{δ}-(4,5-dihydrothiazol-2-yl)-L-ornithine (**17**) and **10**; a lead but non-isoform selective inhibitor, and a highly selective iNOS inhibitor respectively.

ii) Synthesise lead compounds.

iii) Evaluate compounds for potency and isoform selectivity of NOS inhibition.

iv) Design, synthesise and test analogues of lead compounds in order to determine their structure activity relationships.

Research Proposal

Introduction

Target compounds have been designed to possess either the 4,5-dihydrothiazole head group or the thiourea head group and to incorporate molecular features of **10** to increase isoform selectivity.

Important features can be gained by examining the structures and possible modes of binding in the NOS active site.

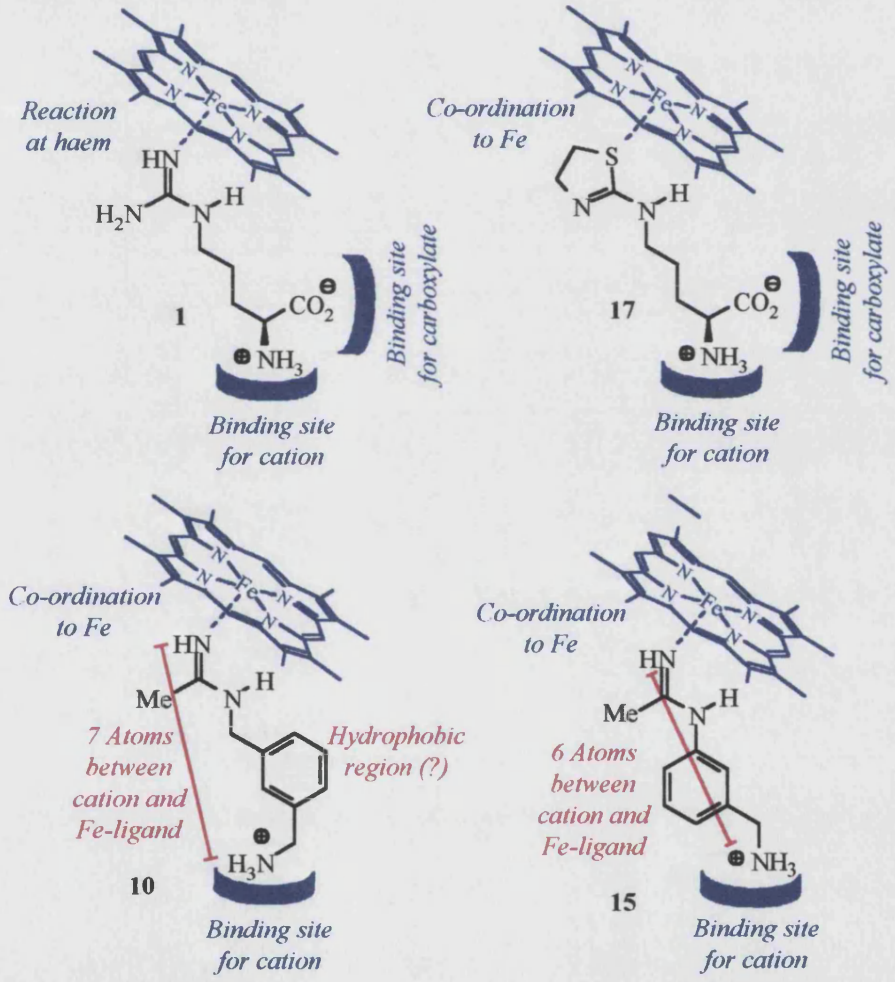


Figure 2.1: Proposed binding of the substrate **1**, a lead non-isoform selective inhibitor **17**, iNOS selective inhibitor **10** and an analogous nNOS selective inhibitor **15**. The diagram illustrates the bridge length theory and shows how the inhibitors may bind to the haem.

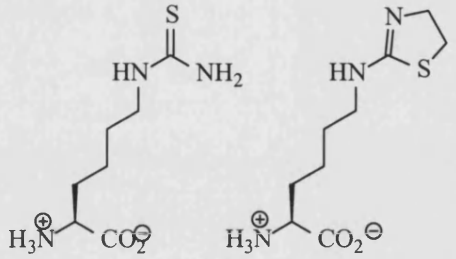
The incorporation of the isothiourea into a five-membered dihydrothiazole ring (as seen in **17**) gave rise to potent but non-selective inhibitors when compared to other aromatic and non-aromatic heterocyclic head groups of L-citrulline analogues (Ulhaq *et al* 1999). The thiourea head group has been shown to give rise to potent NOS inhibitors e.g. thiocitrulline.

Compound **10** is a slow, tight-binding inhibitor, which is highly selective for iNOS and has 7 atoms in the bridge between the remote amidine N and $N^{+}H_3$, whereas the lower homologue of **10** (**15**) has six atoms and displays selectivity for the nNOS isoform. Compound **17** possesses a six atom bridge and displays no isoform selectivity; therefore bridge length appears to be important in the selectivity of the inhibitor.

The benzene ring in compounds **10** and **15** is believed to help the inhibitor maintain the correct conformation of the acetamide that binds to the haem iron and the amine, which binds in the cation pocket (figure 2.1).

The proposal was to synthesise various target compounds related to the structure of **17** and **10**. The following describe the proposed compounds.

Analogues of N^{δ}-(4,5 dihydrothiazol-2-yl)-L-ornithine

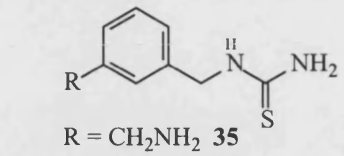


129 **131**

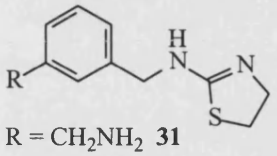
N^{ε}-(4,5-Dihydrothiazol-2-yl)lysine **131** is the chain-extended homologue of the non-selective potent inhibitor **17** and **129** is the chain-extended homologue of the known inhibitor **8**. Both targets were designed to determine whether extending the bridge length to seven atoms would increase the selectivity and potency of the target.

It is proposed that both **129** and **131** will bind in a similar conformation to arginine in the binding site with the sulphur atom in the head groups co-ordinating to the iron atom in the haem. The synthesis of the two lysine derivatives allows a direct comparison to be made between the two different head groups.

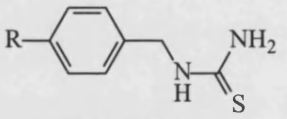
Analogues of 1400W



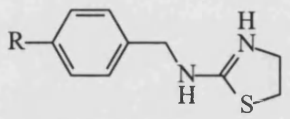
R = CH_2NH_2 **35**
OMe **110**
CO_2H **96**



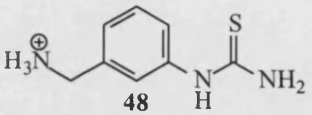
R = CH_2NH_2 **31**
OMe **112**
CO_2H **99**



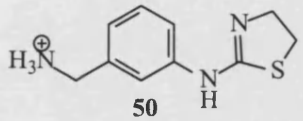
R = CH_2NH_2 **40**
OMe **114**
CO_2H **104**



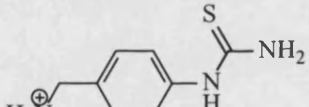
R = CH_2NH_2 **42**
OMe **21**
CO_2H **107**



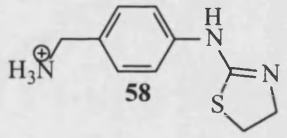
48



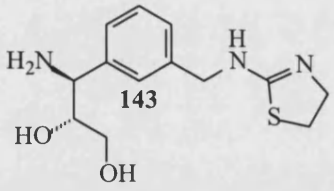
50



56



58



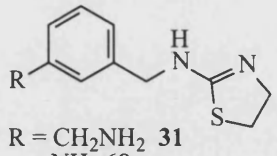
143

The properties and the position of different functional groups around the aromatic ring play important roles in determining potent and selective NOS inhibitors. To examine these effects targets were synthesised with OMe, NH_2 or CO_2H functionality in both meta and para relationships between the groups. The synthesis of the above targets will allow direct comparisons to be made between the functional groups, the ring positions and the head groups and help understand more about the SAR.

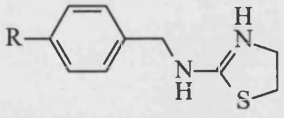
The reason behind the synthesis of **143** was that it possesses the dihydroxyethyl motif, which was shown to increase selectivity for iNOS by 700 fold in N-(5(S)-amino-6,7-dihydroxyheptyl)ethanimidamide dihydrochloride (**18**) versus L-NIL (**5**) (Hallinan *et al* 1998). Hallinan *et al* have shown that chirality plays a direct role in the activity of the inhibitor. The diasteroisomer of **18** showed a reduction of four times the potency as an iNOS inhibitor. Compound **143** was synthesised to incorporate all the molecular features suggested by SAR to be important for iNOS inhibition.

Discussion

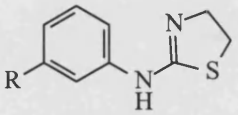
Synthetic Targets



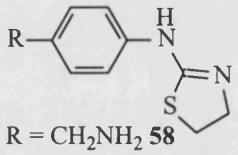
R = CH_2NH_2 **31**
NH_2 **68**
OMe **112**
CO_2H **99**



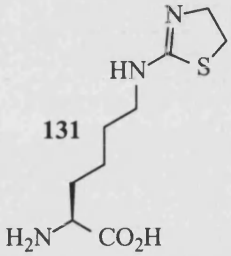
R = CH_2NH_2 **42**
NH_2 **75**
OMe **21**
CO_2H **107**



R = CH_2NH_2 **50**
OMe **119**
CH_2CO_2H **82**



R = CH_2NH_2 **58**
OMe **123**
CH_2CO_2H **89**



131

The target compounds above were designed to retain the dihydrothiazole head group as Ulhaq *et al* 1999 showed that target NOS inhibitors with this head group showed increased potency compared to those with the thiazole head group and other atom changes within the five-membered ring.

	IC_{50} μM (rat iNOS)	% inhibition at 100 μM (rat iNOS)
N^{δ}-(4,5-Dihydrothiazol-2-yl)-L-ornithine	8.1	-
N^{δ}-(Thiazol-2-yl)-L-ornithine	199	-
N^{δ}-(4,5-Dihydrooxazol-2-yl)-L-ornithine	-	18 %
N^{δ}-(4-Methylthiazol-2-yl)-L-ornithine	-	26 %

Table 3.1: IC_{50} values for different ornithine derivatives. Values taken from Ulhaq *et al* 1999.

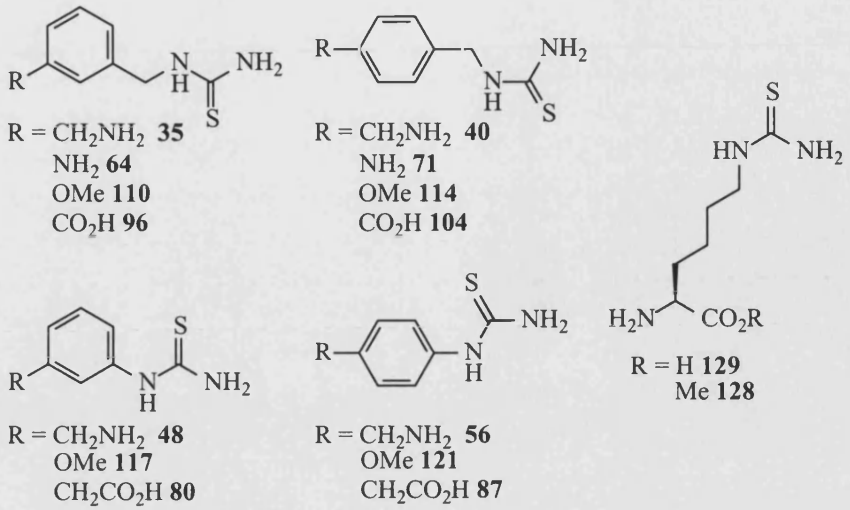
Table 3.1 clearly shows that the dihydrothiazole head group is twenty-five-fold more potent than the aromatic thiazole. The addition of the methyl substituent on the thiazole diminishes the potency of the inhibitor quite considerably, as does the replacement of the sulphur atom with oxygen. The results show that sulphur plays an important role in the head group functionality of the NOS inhibitor; this may be due to the binding interactions with the haem iron. The sulphur atom in **17** is a softer

nucleophile and therefore, a better ligand for binding to iron than the oxygen in the oxazoline.

Compound **17** showed potency but not isoform selectivity for either cNOS or iNOS (IC_{50} 1.3 μM and 8.1 μM, respectively). The chain length is an important characteristic of selective NOS inhibitors, as shown by the two arginine analogues **5** and **6**. Compound **5** has a four-carbon chain between the amino acid moiety and the acetamidine head group and displays selectivity for iNOS over cNOS while **6** exhibits no selectivity for either isoform. N^{ε}-(4,5-Dihydrothiazol-2-yl)lysine dihydrochloride (**131**) was synthesised to retain the dihydrothiazole head group while increasing the carbon chain length to four atoms and so give rise to a potentially selective and potent iNOS inhibitor.

The aromatic targets (**31, 50, 68, 42, 58, 75, 112, 119, 21, 123, 82, 99, 107, 89**) were based on the known selective iNOS inhibitor **10**. The benzene ring is believed to help maintain the inhibitor in the correct conformation for binding to the active site. As mentioned above, the number of atoms between the head group and either the remote amine (as in **10**) or the amino acid functionality (as in **5** and **6**) appears to be highly significant in the synthesis of selective NOS inhibitors. Target **31** was synthesised to maintain the seven-atom bridge and the same remote amine functionality as seen in **10** but possessing the dihydrothiazole head group. The lower homologue of **10** (**15**), which has a six-atom bridge length between the head group, and the remote amine group gave rise to a nNOS selective inhibitor (Collins *et al* 1998). Target **50** was synthesised to be the lower homologue of **31,** testing this theory of the bridge length on the selectivity of NOS inhibition.

Targets **42** and **31** are analogous to **58** and **50**. These targets were synthesised to compare the effects of the potency and selectivity in NOS inhibition in relation to the head group functionality. Both targets **75** and **68** have the same bridge length as **58** and **50**, but with the remote amine group directly attached to the benzene ring. These four targets could then be evaluated to ascertain whether the optimum position for the methylene unit was directly attached to the ring or to the dihydrothiazole head group. The same theory was applied to targets **99, 107, 82** and **89** which possessed the carboxyl functionality.



Different functional groups were synthesised onto these targets e.g. NO_2, NH_2, OMe, CO_2H, to examine the different roles played by electron-withdrawing groups, electron-donating groups and the size of the group on the potency and selectivity of the targets. Targets were synthesised with both the meta and para relationships between the groups so that a direct comparison could be made between both the functional groups and the ring positions.

The second group of targets (illustrated above) were designed to be analogous with the dihydrothiazole targets but with differences in the head group functionality. Citrulline (one of the products of the NOS reaction) is not a good inhibitor of the reaction but, when the ureido oxygen is replaced by sulphur to give **8**, the characteristics of the inhibitor change dramatically. Compound **8** is fairly potent but non-selective inhibitor of NOS (table 3.2). Other NOS inhibitors have been synthesised with the thiourea head group these include S-ethylisothiourea and S-isopropylisothiourea (Garvey *et al* 1994) and aromatic targets such as S-ethyl N-phenylisothiourea (Shearer *et al* 1997a).

	IC_{50} μM		Ki μM	
	iNOS	cNOS	eNOS	nNOS
Thiocitrulline	4.6[a]	1.7[a]		
S-ethyl N-phenylisothiourea	0.87 (Ki μM)[b]	-	0.40[b]	0.12[b]
S-ethylisothiourea	0.019[c]		0.039[c]	0.029[c]

Table 3.2: IC_{50} values for different thiourea derivatives. a Ulhaq *et al* 1999, b Shearer *et al* 1997a, c Garvey *et al* 1994.

The lysine target **129** is an analogue of **8** possessing the extra CH_2 unit in the chain length, which will hopefully increase the selectivity of the target but retain the potency seen in **8**. Target **128** is the methyl ester of **129** and was synthesised to see if increasing the steric bulk around the carboxylate had any affect on the potency and selectivity of the inhibitor as seen in **7** and **3**. Compound **7** being more selective for cNOS over iNOS (ref. table 1.2 chapter 1). Also the effect of changing the overall charge of the inhibitor from negative (without the methyl ester) to neutral can be examined.

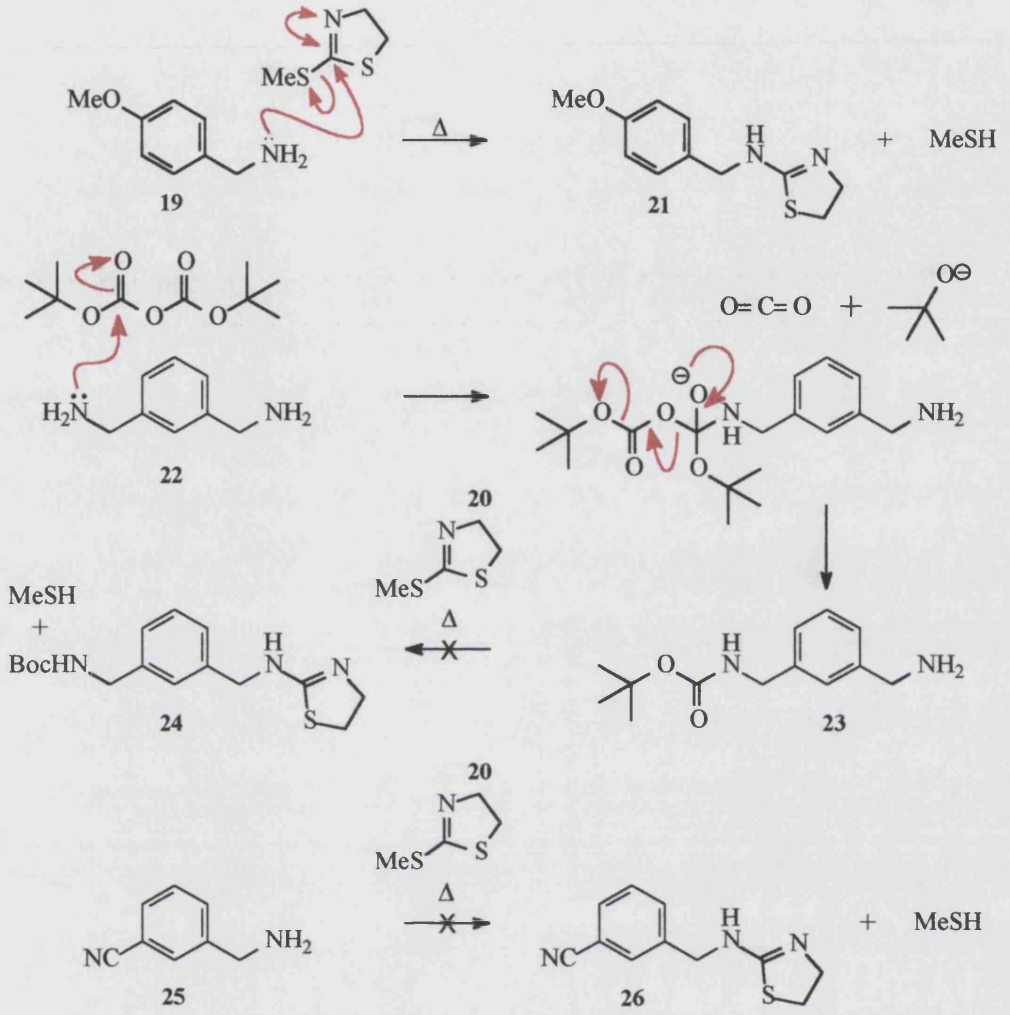
The same hypotheses (explained earlier) that were applied to targets **31, 50, 68, 42, 58, 75, 112, 119, 21, 123, 82, 99, 107, 89** about the benzene ring being important for the inhibitor conformation and the bridge length between the head group and the functional group were applied to targets **35, 48, 64, 40, 56, 71, 110, 117, 114, 121, 80, 96, 104, 87**. Comparisons between functional groups and the position around the aromatic ring can be made as before with the dihydrothiazole targets but also the effects that the different head groups have on NOS inhibition can be examined.

The first step in the synthesis of all the targets was to devise an efficient and high yielding method for synthesising both the dihydrothiazole head group and the thiourea head group.

Strategies towards the synthesis of the dihydrothiazole head group

Target **21** was the first target to be synthesised. Stokker *et al* 1982 have reported a method for preparing 2-amino-4,5-dihydrothiazoles from primary amines by an addition-elimination reaction with 2-methylthio-4,5-dihydrothiazole in ethanol at reflux. This procedure would be extremely beneficial to the synthesis of these targets as the head group functionality would be added in a one-step reaction, dramatically decreasing the number of steps in the overall synthesis. The starting material 4-methoxybenzylamine (**19**) was chosen as a model for **21**. However, when this procedure was applied to the model no reaction took place. It was necessary to heat a mixture of the reagents at 180°C for 4 h in the absence of solvent to achieve a 30% yield of the desired model product **21** as a crystalline solid.

Retrosynthetic analysis suggested that a common starting material for target **31** would be 1,3-bis(aminomethyl)benzene (**22**). Since only one of the amines needed to be converted into the various head groups, the other needed to be protected. The Boc group is one of the most frequently used groups to protect amino compounds. It is inert towards catalytic hydrogenolysis and resistant towards basic and nucleophilic reagents but is readily cleaved in high yield by strong acids. Therefore, this makes it a very suitable protecting group for the later steps in the synthesis. Diamine **22** was treated with 0.3 equivalents of di-*tert*-butyl dicarbonate, giving a mixture of the mono-Boc diamine **23** and the starting material. These were readily separated, as the diamine **22** is highly soluble in water; the crystalline product **23** was obtained in excellent yield (78%). The selectively Boc protected amine was then treated with 2-methylthio-4,5-dihydrothiazole at reflux for 4 h at 180°C. However, the conditions were obviously too harsh for this compound, as no products could be identified from the ^{1}H NMR spectrum of the crude reaction mixture. This method was then applied to a different starting material: 3-cyanobenzylamine with the expectation that the cyano group would be able to withstand the harsh conditions. Once again ^{1}H NMR results showed extensive decomposition. Therefore, another method was needed to add the dihydrothiazole head group onto the amine (Scheme 3.1).

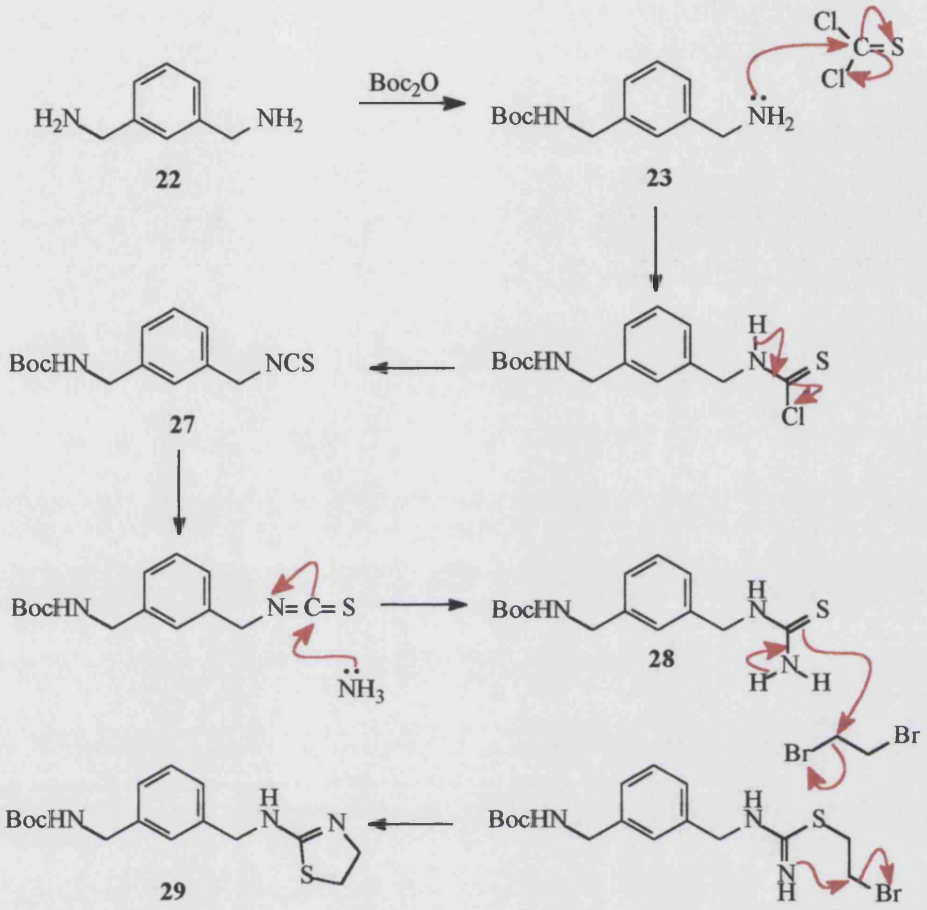


Scheme 3.1: Synthetic routes for the dihydrothiazole head group.

Another approach to the synthesis of 2-substituted dihydrothiazoles is the classical Gabriel synthesis (Metzger 1984), which involves condensation of the thioamide with 1,2-dibromoalkanes to give the corresponding 4,5-dihydrothiazoles. This method of synthesising the dihydrothiazole targets would be advantageous because one of the intermediate steps involves the synthesis of thioureas.

The dihydrothiazoles can be synthesised from thioureas which in turn can be synthesised from thiocyanates. The isothiocyanate can easily be prepared from the mono-Boc protected diamine (**23**) by treatment with thiophosgene in the presence of $CaCO_3$ to prevent the mixture from turning acidic and therefore cleaving the

protecting group. The unprotected amine acts as the nucleophile and attacks the carbon of the thiophosgene and the reaction proceeds by an addition elimination mechanism. The thiourea can be disconnected by the "cleavage" of the terminal C-N bond. Ammonia represents the strong nucleophile, which is bubbled through a solution of the electrophilic isothiocyanate in chloroform to give the thiourea in excellent yield (97%). The mechanism for the next step in the reaction is proposed in scheme 3.2. This involves nucleophilic attack by the sulphur on 1,2-dibromoethane, which displaces one of the bromine atoms. The initial step is followed by cyclisation and the loss of the other bromine atom. Ulhaq *et al* 1999 used this method for the addition of the C_2 unit on ornithine derivatives and only achieved moderate yields (35%) for the target dihydrothiazole. When this procedure was carried out on the thiourea **28** even after repeated attempts, the yields was exceptionally low (3%) so, therefore, a more efficient alternative method was required (Scheme 3.3).



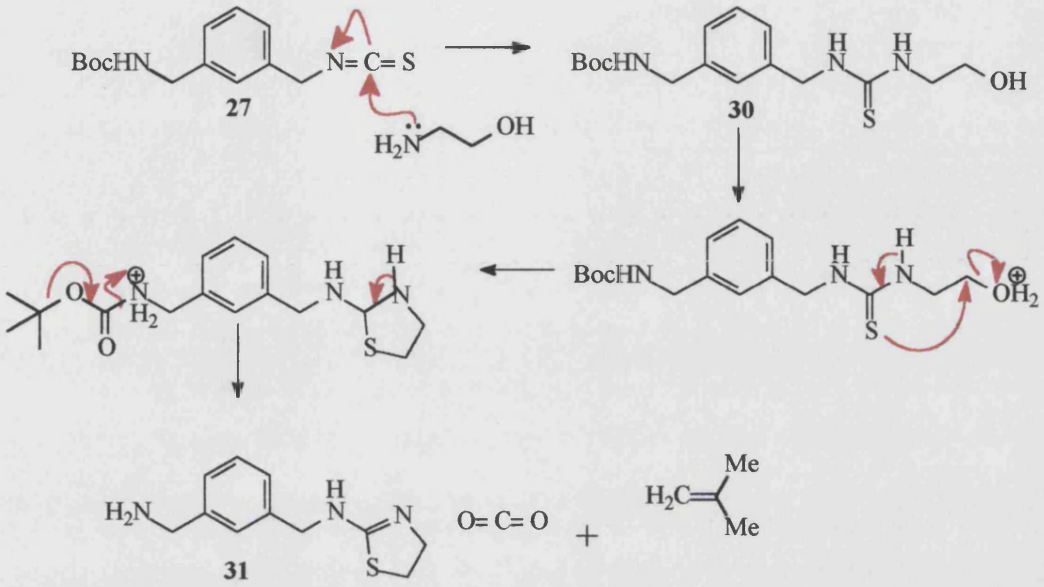
Scheme 3.2: Synthetic route to the dihydrothiazole head group using the Gabriel synthesis.

An alternative method for the synthesis of dihydrothiazoles has been reported by Caujolle *et al* 1989. An isothiocyanate in acetone added dropwise over 30 min to a solution of 2-aminoethanol in acetone and refluxed for 4-5 h produces a thiourea intermediate. The N'-(2-hydroxyethyl)thiourea intermediate is then cyclised by treatment with boiling aq. hydrochloric acid (6 M) for 40 h to give the corresponding dihydrothiazole.

This procedure was tried on the isothiocyanate intermediate **27**. The amine of the 2-aminoethanol is more nucleophilic than the oxygen and so readily reacts with the electrophilic carbon to add the two-carbon unit onto the thiocyanate. This method successfully gave the intermediate **30** in a reasonable yield (37%).

Cyclisation of **30** was achieved by treatment with boiling aq. hydrochloric acid (6 M) for 40 h. The progress of the reaction was monitored by ^{1}H NMR. The crude ^{1}H NMR spectrum after 24 h showed complete removal of the Boc protection but a mixture of dihydrothiazole and 2-hydroxyethyl units. Protonation of the OH group makes it a good leaving group; nucleophilic attack by the sulphur at the terminal CH_2 and expulsion of water leads to cyclisation. Yields in repeated runs were *ca.* 70%. An advantage of this method is that the target **31** is isolated directly by evaporation as the hydrochloride salt, so achieving two steps in one; the cyclisation and the removal of the Boc protecting group. 3-Cyanobenzylamine would also have been a suitable starting material for this synthesis, with the reduction of the cyano group (with Pd/C) being the last step in the synthesis. However, there is the risk that the cyano group would be hydrolysed to a carboxamide or a carboxylic acid under these conditions. The addition and removal of the Boc protecting group were high yielding steps in the synthesis and so **22** was deemed the more appropriate starting material.

Subsequently, it was found that treatment of the N'-(2-hydroxyethyl)thiourea with trifluoroacetic acid also produced the cyclised trifluoroacetic acid salt in approximately ten minutes (Kocienski 1994) (Scheme 3.3). Trifluoroacetic acid is a stronger acid than hydrochloric acid due to the electron-withdrawing properties of fluorine; but the differences in their dissociation constants alone would not be enough to increase the rate of reaction sufficiently to complete the cyclisation in ten minutes; therefore there must be other factors involved. An additional explanation is that trifluoroacetic acid reacts with the hydroxyethyl thiourea (**30**) to form the trifluoroacetate ester which is a much better leaving group that OH and so the cyclisation occurs at a much faster rate than with aq. hydrochloric acid (scheme 3.6).

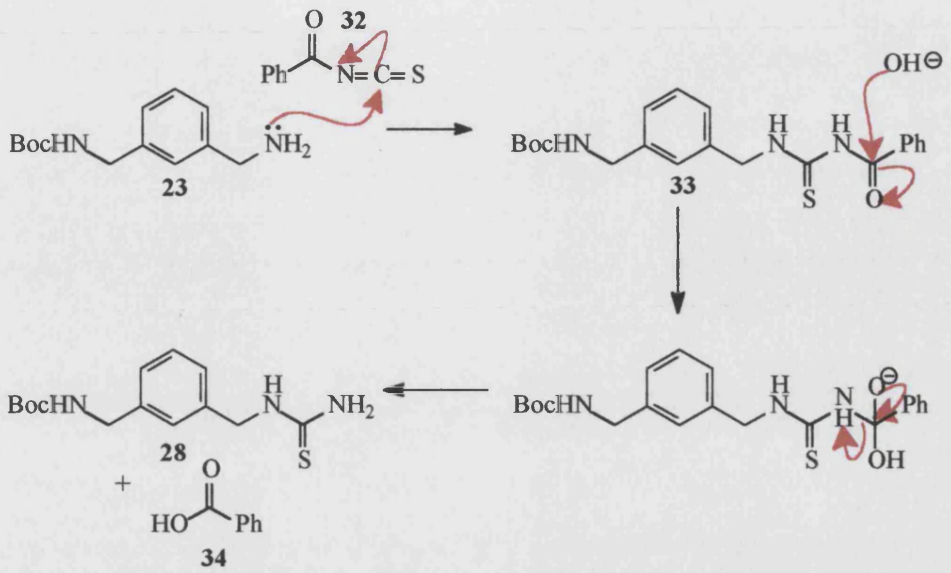


Scheme 3.3: Reaction mechanism for the formation of **31**.

Strategies towards the synthesis of the thiourea head group

Retrosynthesis shows that the thiourea head group can be disconnected by the cleavage of either the terminal C-N bond or the other C-N bond. Disconnection of the terminal C-N bond would require a strong electrophile and a nucleophile. An ideal compound for the electrophile would be an isothiocyanate with ammonia representing the nucleophile. As seen in scheme 3.2, these reactions have already been achieved in high yields in the synthesis of the dihydrothiazole head group, using **22** as the starting material. The advantage of using this synthetic route is that the intermediate thiocyanates generated can be used in the synthesis of the dihydrothiazole targets as well. This was the method chosen to synthesise targets with the thiourea head group.

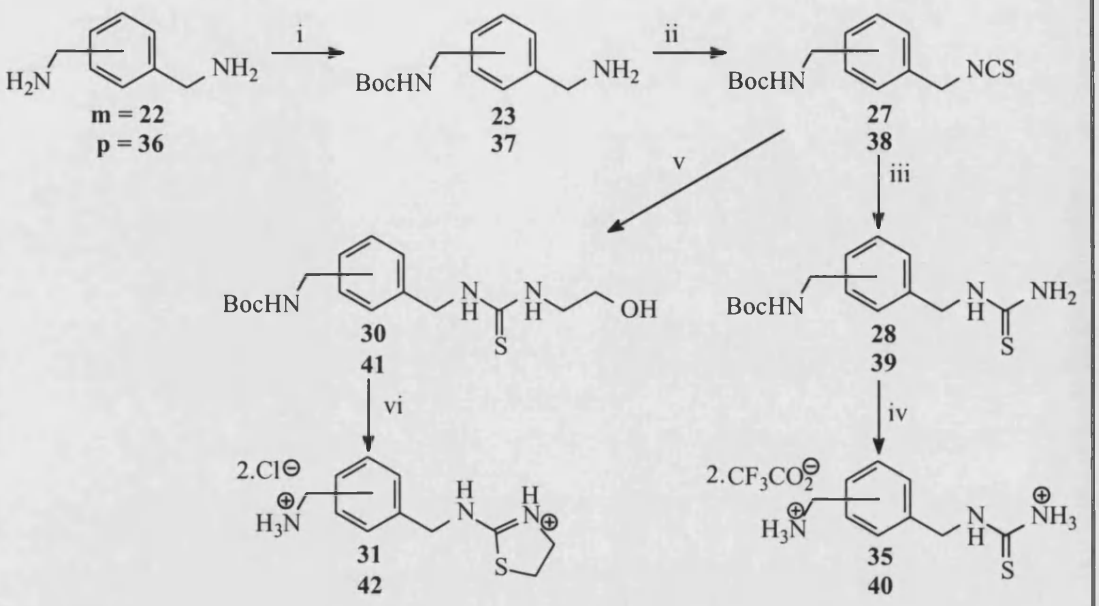
Disconnection of the other C-N bond would require benzoyl isothiocyanate (**32**) as the electrophile which reacts with the mono-Boc protected diamine **23** to give the intermediate 1,1-dimethylethyl N-(3-(N'-benzoylthioureidomethyl) phenylmethyl)carbamate. Treatment of the intermediate with alkaline hydrolysis gives the corresponding 1,1-dimethylethyl N-(3-(thioureidomethyl)phenylmethyl)carbamate **28**.



Scheme 3.4: Alternative synthetic pathway for the synthesis of **28.**

The above targets were designed and synthesised so that a range of structural effects could be correlated to NOS inhibition. This would enable conclusions to be drawn about the effects of the head group (dihydrothiazole and thiourea), the position, type and size of the functional group on NOS potency and selectivity. Also information can be gained about the structural features, which are important for the inhibitor to bind to the active site of the enzyme and so increase understanding of structure activity relationships.

Synthetic routes to targets 31, 35, 40 and 42



Scheme 3.5: Synthesis of targets **31, 35, 40** and **42.** Reagents and conditions: i) Boc_2O, CH_2Cl_2, Et_3N, 16 h; ii) $CSCl_2$, $CHCl_3$, 16 h; iii) NH_3, CH_2Cl_2; iv) CF_3CO_2H, 5 min; v) 2-aminoethanol, acetone, reflux; vi) aq. HCl 6 M, reflux 40 h.

Synthetic routes to targets are given in scheme 3.5. Retrosynthetic analysis suggested that a common starting material for the meta-substituted targets would be 1,3-bis(aminomethyl)benzene (**22**) and 1,4-bis-(aminomethyl)benzene (**36**) for the para-substituted targets. For both targets, selective protection of one of the amine groups was required and this was again carried out using the Boc protecting group. The Boc protecting group is inert towards catalytic hydrogenolysis and resistant towards basic and nucleophilic reagents but is readily cleaved in high yield by strong acids.

A surprising result from the ^{1}H NMR spectral analysis of **23** is that the CH_2NH_2 singlet peak was seen at δ 4.34, while the CH_2NHBoc doublet peak appeared at δ 3.90. The electron-withdrawing properties of the carbonyl usually influence the CH_2 next to the NHBoc, pushing the signal further downfield so that the CH_2NH_2 is usually seen further upfield of δ 4.34. The ^{1}H NMR spectrum peaks must be correctly assigned as the NH attached to the Boc protecting group is coupled into the CH_2 and therefore seen as a doublet and not a singlet. Protons belonging to free

amine substituents do not usually couple to neighbouring groups, which explains why the signal seen is a singlet and not a doublet.

The reaction of **23** with thiophosgene gave the desired isothiocyanate **27**, this was achieved in reasonable yield (37%). The mechanism of this (and subsequent steps) reaction were explained earlier in the chapter. IR spectral analysis showed that the characteristic strong peak of the isothiocyanate had been formed at 2060 cm^{-1}. The formation of the novel thiourea **28** was characterised by the disappearance of the isothiocyanate peak in the IR spectrum and the appearance of the C=S peak at 1164 cm^{-1}. Deprotection of the thiourea **28** was carried out in trifluoroacetic acid. Compound **27** was stirred in trifluoroacetic acid for five minutes to give the corresponding bis(trifluoroacetate) salt in a quantitative yield. The cyclisation of the 2-hydroxyethyl thiourea intermediate **30** was carried out under reflux for 36 h in aq. hydrochloric acid (6 M). The novel highly polar product **31** was achieved in good yield (47%). ^1H NMR spectra analysis showed that the dihydrochloride salt had formed as an N^+H_3 peak and two NH peaks were seen at δ 8.70 and 10.89, respectively.

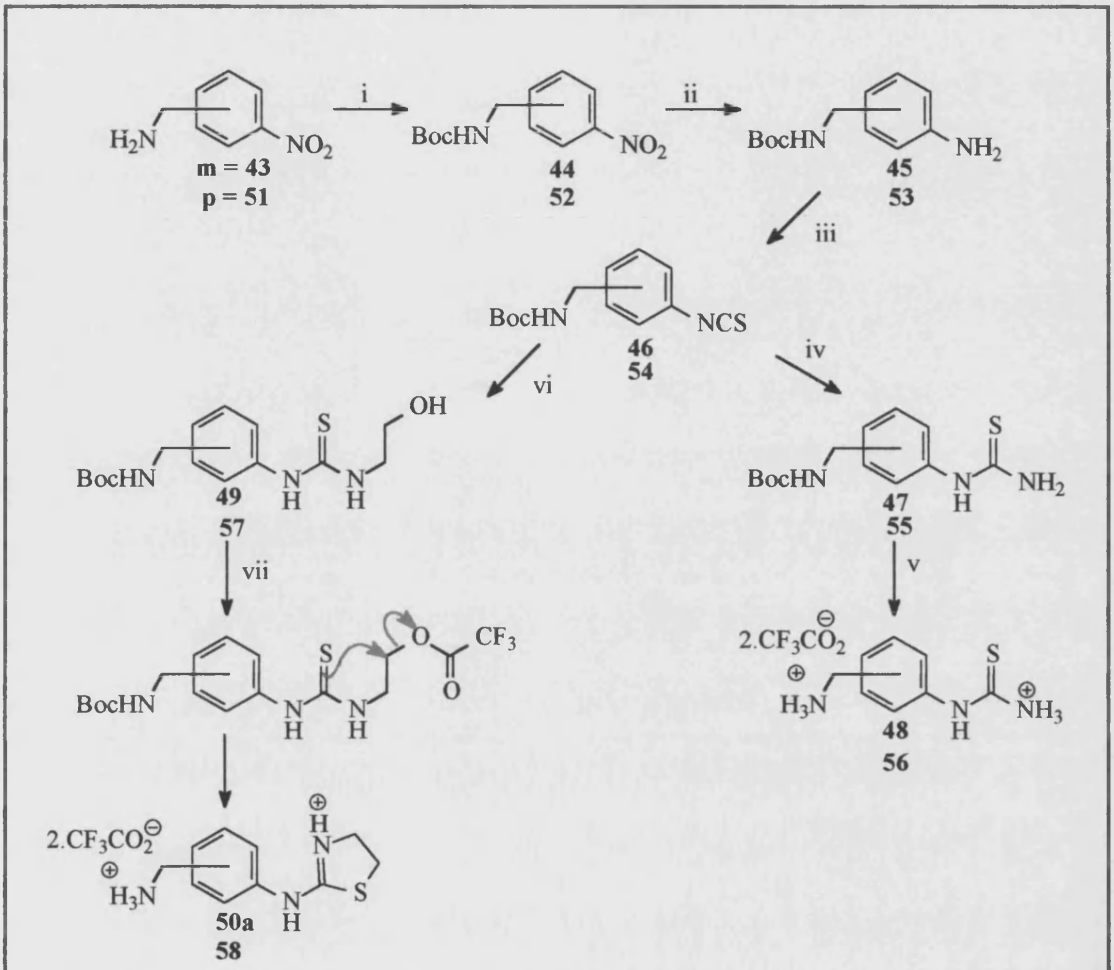
Para-substituted target compounds **40** and **42** were synthesised in the same way as the meta-substituted targets. Boc protection of **36** was achieved in 80% yield. ^1H NMR spectra analysis showed that the doublet signal corresponding to CH_2NHBoc was seen further downfield of the CH_2NH_2 as expected. The novel isothiocyanate and thiourea intermediates **38** and **39** were achieved in good yields of 80% and 71% respectively as crystalline solids. The removal of the Boc protecting group from **39** was carried with trifluoroacetic acid giving rise to the corresponding target salt in a quantitative yield.

Cyclisation of **41** was carried out under the same conditions as **31** (reflux for 36 h in aq. hydrochloric acid (6 M)), but difficulties occurred with the purification of **42**. Attempts were made to purify the product by recrystallisation and the re-addition of the protecting group to the cyclised product in the hope that the compound could

then be purified by column chromatography. After repeated attempts target **42** was achieved by recrystallisation from propan-2-ol.

Synthetic routes to targets 48, 50, 56, 58, 64, 68, 71 and 75

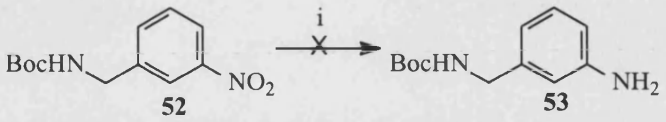
The synthetic routes to target compounds **48**, **50, 56** and **58** are given in Scheme 3.6.



Scheme 3.6: Synthesis of targets **48, 50, 56** and **58**. Reagents and conditions: i) Boc_2O, CH_2Cl_2, Et_3N, 16 h; ii) $SnCl_2.2H_2O$, EtOH, reflux; iii) $CSCl_2$, $CHCl_3$, 16 h; iv) NH_3, CH_2Cl_2; v) CF_3CO_2H; vi) 2-aminoethanol, acetone, reflux; vii) CF_3CO_2H.

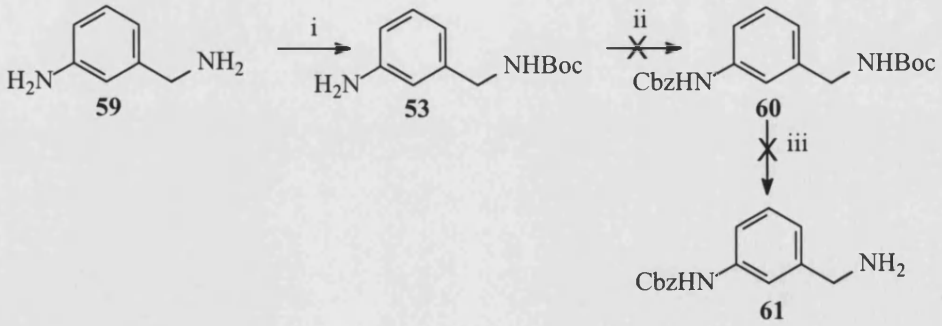
Retrosynthetic analysis suggests that a common starting material for the meta-substituted targets would be 3-nitrobenzylamine (**43**) and 4-nitrobenzylamine (**51**) for the para-substituted targets. Before the synthesis of the head groups could be carried out, the amine needed to be protected and the nitro group reduced to the amine. Initially, the reduction was carried out with hydrogen and Pd/C but, after repeated attempts, no significant yields were achieved, and so another method was needed (Scheme 3.7). Bellamy *et al* 1984 proposed another approach to the reduction of the nitro group, in which the reduction could occur under neutral

conditions. This was essential to the reaction otherwise the Boc protecting group would be cleaved. The protected nitro compound **52** was added to 2.8 equivalents of $SnCl_2$ in ethanol and boiled for 30 min. The mixture was made basic, subsequently extracted and evaporated to give the amine **53** in moderate yield (31%).



Scheme 3.7: Synthesis of **53**. Reagents and conditions: i) Pd/C, H_2.

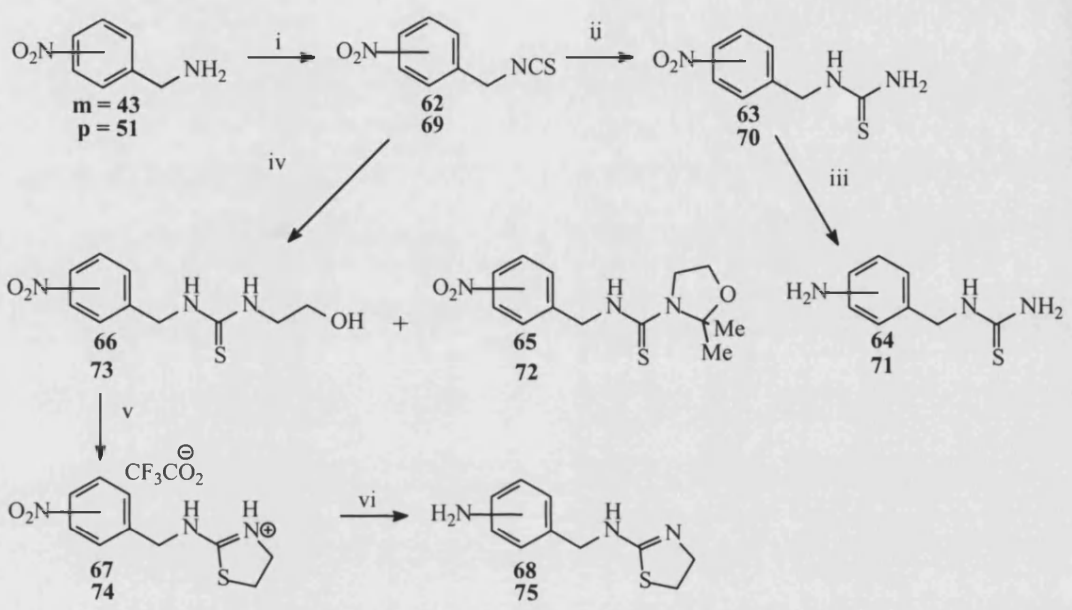
The synthetic route for targets **64, 68, 71** and **75** is given in scheme 3.9. The first steps in the synthesis was to orthogonally protect the amine groups in the starting material 3-(aminomethyl)benzylamine (**59**). The proposal was to use the Cbz and Boc protecting groups. The Boc protecting group would be cleaved under mild acidic conditions to add the head functionality to the target and the Cbz group would be removed in the final step. Failed attempts were made to add the Cbz protecting group to 1,1-dimethylethyl N-(3-aminophenylmethyl)carbamate (**60**) (this is shown in scheme 3.8). An alternative method was required to achieve target compounds **64, 68, 71** and **75**.



Scheme 3.8: Synthesis of **61**. Reagents and conditions: i) Boc_2O, CH_2Cl_2, 16 h; ii) Benzyl chloroformate, CH_2Cl_2, Et_3N, 16 h; iii) aq. HCl.

As mentioned earlier nitro groups can be reduced in the absence of base or acid using $SnCl_2$. Reducing a nitro group without using palladium meant that the reaction could

be carried out in the presence of the sulphur atom. As a consequence the reduction of the nitro group could be the final step in the synthesis after the head group functionality had been assembled. The starting materials were changed to 3-nitrobenzylamine (**43**) and 4-nitrobenzylamine (**51**) and the targets were synthesised as shown in scheme 3.9.

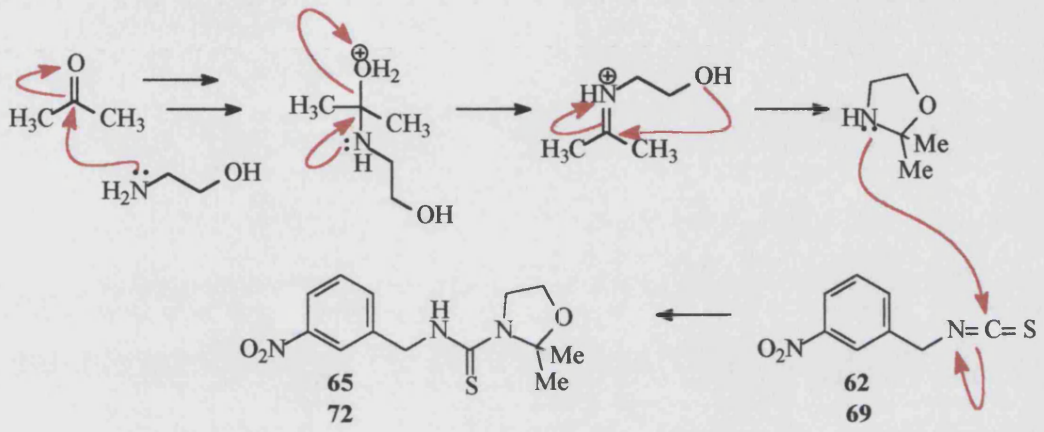


Scheme 3.9: Synthesis of targets **64, 68 71** and **75**. Reagents and conditions: i) $CSCl_2$, $CHCl_3$, 16 h; ii) NH_3, CH_2Cl_2; iii) $SnCl_2.2H_2O$, EtOH, reflux; v) CF_3CO_2H; iv) 2-aminoethanol, acetone, reflux; v) CF_3CO_2H; vi) $SnCl_2.2H_2O$, EtOH, reflux.

Additional compounds (**65** and **72**) were isolated when 2-aminoethanol was added to the intermediates **62** and **69** respectively. The minor tetrahydrooxazole products were less polar than the desired major products **66** and **73** and so were easily separated by column chromatography. Confirmation of the oxazole products was achieved with ^1H NMR spectra and FAB+ mass spectra. The ^1H NMR spectrum showed triplet signals corresponding to the oxazole CH_2 groups at δ 3.82 and 4.05, and the appearance of the two methyl signals at δ 1.80. The FAB+ve ion mass spectrum showed the [M + H] peak at 296.

Tetrahydrooxazoles **65** and **72** were only formed when the nitro functionality was directly attached to the benzene ring. The nitro group is a very strong electron

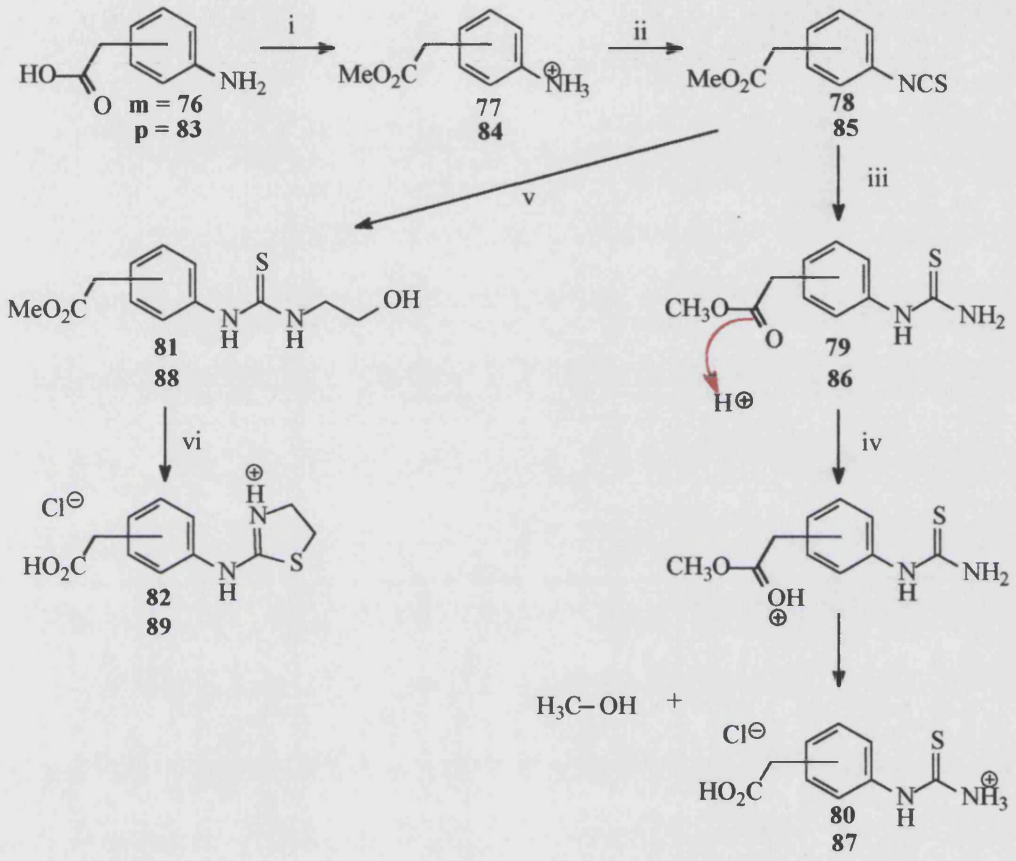
withdrawing group and so pulls electrons away from the benzene ring, making the isothiocyanate carbon more electrophilic. The proposed mechanism for this reaction involves the reaction solvent (acetone) and 2-aminoethanol reacting together to form the nucleophilic species, which then attacks the electrophilic carbon (scheme 3.10).



Scheme 3.10: Mechanism for the synthesis of oxazole compounds **65** and **72**

Synthetic routes to targets 80, 82, 87, 89, 96, 99, 104 and 107

The starting materials used for the targets with the head group directly attached to the ring (**89, 82, 87, 80**) were 3-aminophenylacetic acid (**76**) and 4-aminophenylacetic acid (**83**) (scheme 3.11).



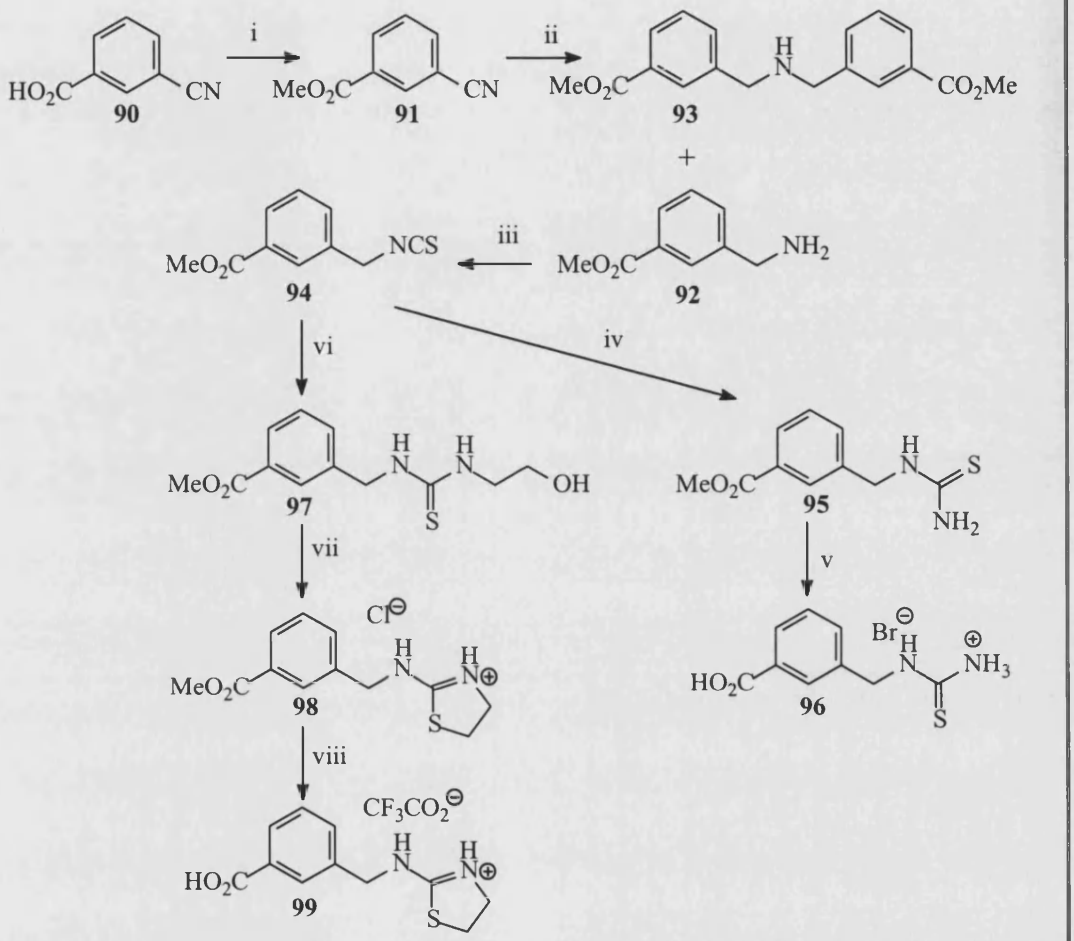
Scheme 3.11: Synthesis of targets **80, 82, 87** and **89**. Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) $CSCl_2$, $CHCl_3$, 16 h; iii) NH_3, CH_2Cl_2; iv) aq. HCl 1 M, 9 d (**80**) aq. HCl 6 M 16 h (**87**); v) 2-aminoethanol, acetone, reflux; vi) aq. HCl 6 M, 40 h, reflux (**82**), aq. HCl 6 M, 16 h (**89**).

The initial step in the synthesis of these targets was to protect the carboxylate functionality. This was carried out for two reasons; i) to increase the solubility of the products ii) to prevent any minor compounds being formed. This is especially important when treating the unprotected starting material with thiophosgene as acid chlorides may be formed. The carboxylate in both starting materials were protected with a methyl ester and the products (**77** and **84**) were achieved in quantitative yields.

Methyl 3-isothiocyanatophenylacetate (**78**) and methyl 3-thioureidophenylacetate (**79**) are both novel compounds and were made in very good yields 77% and 99% respectively. The removal of the methyl ester from **79** was carried out in aq. hydrochloric acid 1 M and the known product **80** was achieved in a good crystalline yield (86%).

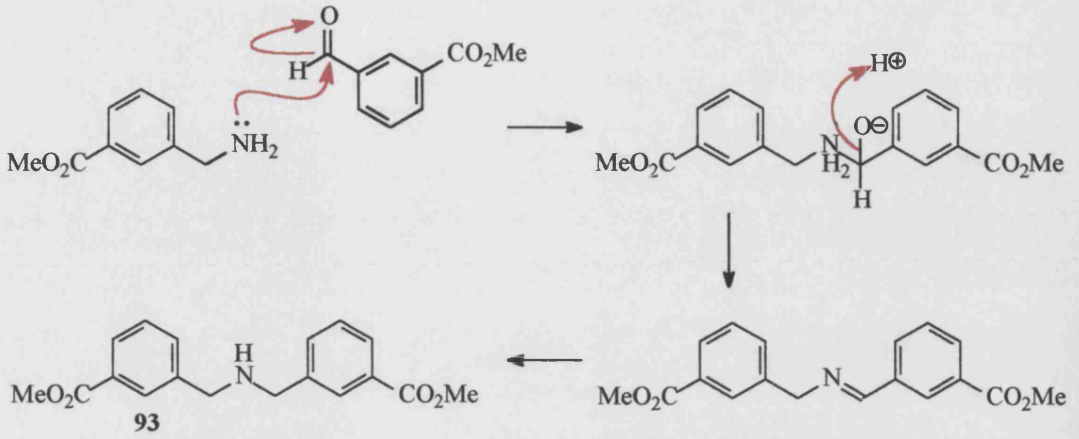
Cyclisation of **81** was carried out in reflux with aq. hydrochloric acid. The removal of the methyl ester from **86** to achieve the thiourea target **87** was achieved by stirring in aq. hydrochloric acid (6 M) for 16h. Interestingly, the removal of the methyl ester to achieve the other dihydrothiazole target (**89**) was carried out in aq. hydrochloric acid (6 M) at room temperature.

3-Cyanobenzoic acid (**90**) was the most suitable starting material for targets **96** and **99** (scheme 3.12).



Scheme 3.12: Synthesis of targets **96** and **99**. Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) Pd/C H_2, MeOH, 16 h; iii) $CSCl_2$, $CHCl_3$, 16 h; iv) NH_3, CH_2Cl_2; v) hydrobromic acid (50%), 16 h; vi) 2-aminoethanol, acetone, reflux; vii) aq. HCl 6 M, 40 h, reflux, MeOH, $SOCl_2$, 4 d; viii) CF_3CO_2H.

Reduction of **91** with Pd/C (10%) gave rise to both the desired product **92** in reasonable yield and a dimerized side product **93** in an exceptionally low yield. During the reaction a partially reduced intermediate is formed which reacts with the amine to produce an imine. The nitrogen double bond in the imine is then reduced forming **93** (scheme 3.13). Confirmation of the dimer came from mass spectra. FAB+ve ion mass spectrum showed the [M + H] peak at 314.

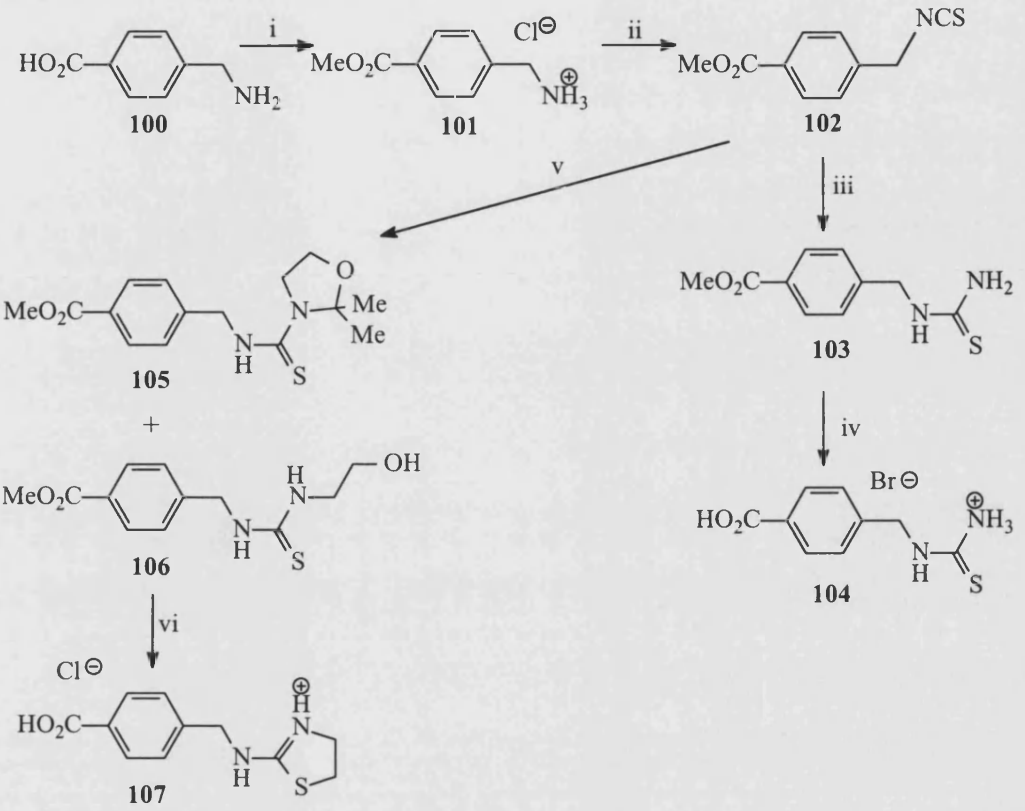


Scheme 3.13: Mechanism for the synthesis of the dimerized side product **93.**

All the targets with the carboxyl functionality were synthesised using methyl ester as the protecting group. However, problems arose when trying to remove the methyl ester when it was directly attached to the ring in the presence of the thiourea head group (**96**). The acid conditions (even aq. hydrochloric acid 1 M) appeared to be too harsh and so removal of the protecting group was tried using 30% NaOH (aq.) (Giordano *et al* 1982), this too was unsuccessful and so hydrobromic acid was tried. Removal of the methyl ester was achieved when **95** was stirred in 50% hydrobromic acid for 16 h.

The final step in the synthesis of methyl 3-(4,5-dihydrothiazol-2-ylamino)benzoate hydrochloride in which **97** was boiled under reflux with aq. hydrochloric acid (6 M) for 40 h gave a product which was difficult to purify. Attempts were made to purify the compound by recrystallisation but a suitable solvent proved hard to find due to the polarity of the compound. The methyl ester of the crude dihydrothiazole was made and deprotected with 50% aq. trifluoroacetic acid (under reflux for 16 h), which yielded the purified product in quantitative yield.

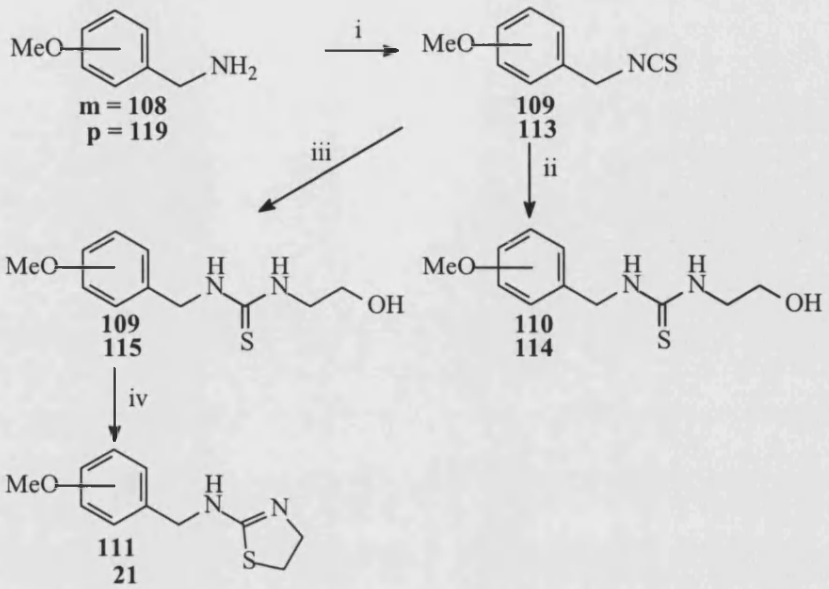
4-Aminomethylbenzoic acid was commercially available and so was used for the synthesis of targets **104** and **107** (scheme 3.14).



Scheme 3.14: Synthesis of targets **104** and **107**. Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) $CSCl_2$, $CHCl_3$, 16 h; iii) NH_3, CH_2Cl_2; iv) hydrobromic acid (50%), 16 h; v) 2-aminoethanol, acetone, reflux; vi) aq. HCl 6 M, 40 h, reflux, MeOH, $SOCl_2$, 4 d.

As seen for the nitro group examples, when the nitro electron-withdrawing group was directly attached to the benzene ring a tetraoxazole was also formed in the reaction with 2-aminoethanol. This also occurred when the carboxyl electron-withdrawing group was in the para position giving rise to compound **105** in reasonable yield (43%). The reaction occurs by the same mechanism as shown in scheme 3.10. Once again the ^{1}H NMR spectra showed the characteristic oxazole triplets at δ 3.64 and 3.96 and the methyl group signal at δ 1.69. The FAB+ve ion mass spectra showed the [M + H] peak at 309.

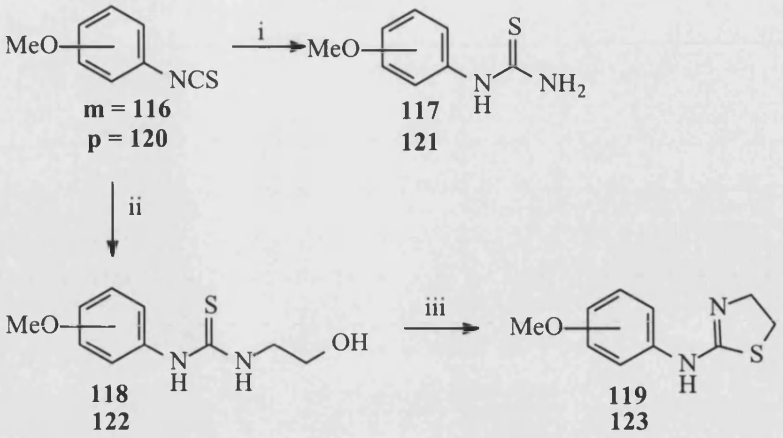
Synthetic routes to targets 21, 110, 112, 114, 117 119, 121 and 123



Scheme 3.15: Synthesis of targets **21, 110, 112** and **114**. Reagents and conditions: i) $CSCl_2$, $CHCl_3$, 16 h; ii) NH_3, CH_2Cl_2; iii) 2-aminoethanol, acetone, reflux; iv) CF_3CO_2H.

The corresponding starting material for targets **112** and **110** was 3-methoxybenzylamine (**108**) and for targets **21** and **114** the starting material was 4-methoxybenzylamine (**19**). Due to the relatively unreactive properties (under the conditions used) of the methoxy group there was no need to add a protecting group to this functionality and so the synthetic pathways for these targets were reduced by several steps (schemes 3.15 and 3.16).

Target **112** was achieved by two different synthetic routes. The 1:1 addition of 3-methoxybenzylamine with 2-methylthio-4,5-dihydrothiazole (mechanism explained in scheme 3.1) was achieved in moderate yield (30%) of crystalline product. When the same target was synthesised by cyclising the intermediate 2-hydroxyethyl thiourea **111** in trifluoroacetic acid, the product was attained in a higher yield (75%) but as an oil. On the basis of this information it would seem that the one-step synthesis gives lower yields but of better purity than the cyclisation carried out in trifluoroacetic acid.



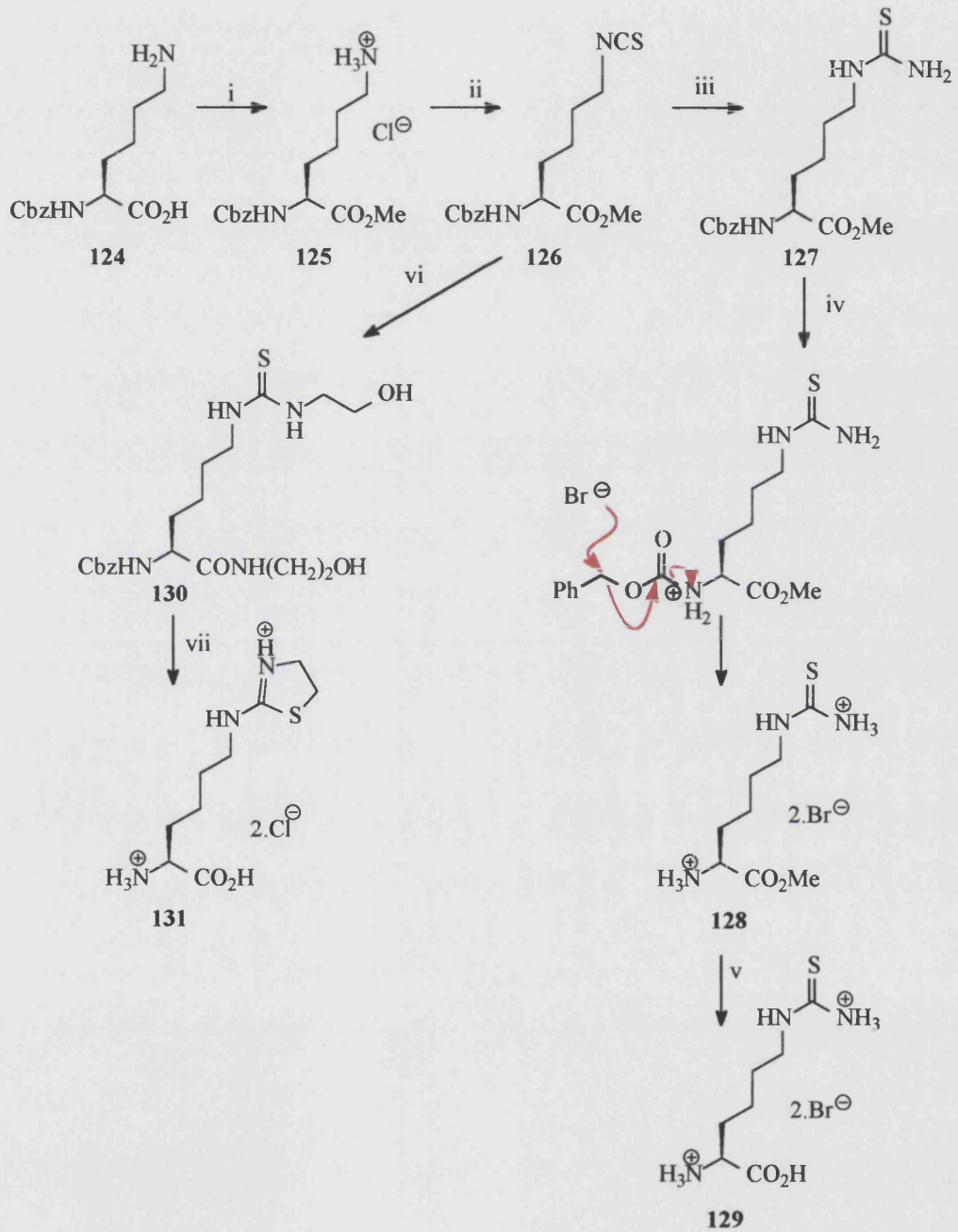
Scheme 3.16: Synthesis of targets **117, 119, 121** and **123**. Reagents and conditions: i) NH_3, CH_2Cl_2; ii) 2-aminoethanol, acetone, reflux; iii) CF_3CO_2H.

The number of steps involved in the synthesis of targets **117, 119, 121** and **123** were very few, as 3-methoxyphenylisothiocyanate (**116**) and 4-methoxyphenylisothiocyanate (**120**) were commercially available and all targets were achieved in one step and were of high crystalline yields (scheme 3.16).

All the steps involved in the synthesis of the methoxy targets were of good yield. In particular, the formation of the thiourea targets **114, 110, 121** and **117** were achieved in exceptional yields (99% or quantitative).

Synthetic routes to targets 128, 129 and 131

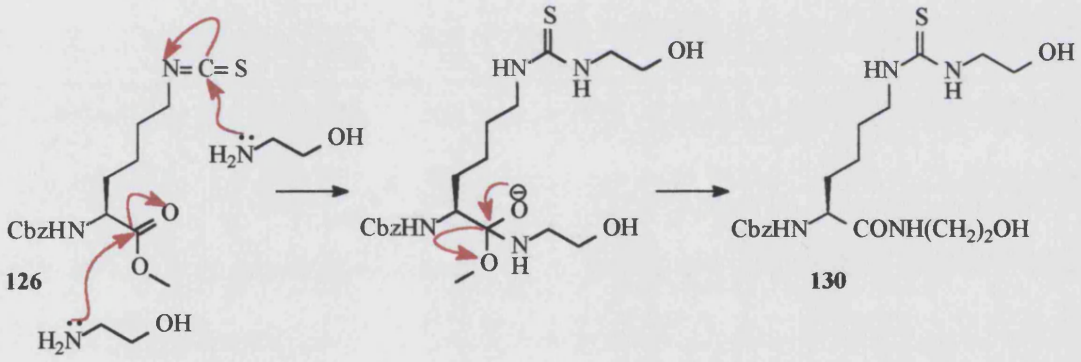
The synthetic route to targets **128, 129** and **131** is given in Scheme 3.17.



Scheme 3.17: Synthesis of targets **128, 129** and **131**. Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) $CSCl_2$, $CHCl_3$, 16 h; iii) NH_3, CH_2Cl_2; iv) HBr in HOAc (17.5%) 15 h; v) hydrobromic acid 16 h; vi) 2-aminoethanol, acetone, reflux; vii) aq. HCl, 6 M 40 h, reflux.

Retrosynthetic analysis suggested that N^{ε}-Cbz-lysine would be an ideal starting material. The carboxyl group was protected with *tert*-butyl ester, which offers a degree of steric shielding. This makes the group resistant to attack by a wide range of nucleophiles (Kocienski 1994). The α-amino group was protected with the most popular amino-protecting group Boc. Cbz groups usually cleave easily with catalytic hydrogenation. However, repeated attempts to remove the ε Cbz group gave no successful unprotected amine. Cbz-Lys-OH (**124**) was also commercially available and so this starting material was used instead. The carboxyl group was then protected as the methyl ester as the method was found to be slightly easier than introducing the butyl ester.

During the penultimate step in the synthesis of **131**, involving **126** reacting with 2-aminoethanol, the methyl ester was exchanged with 2-aminoethanol(scheme 3.18).



Scheme 3.18: Mechanism showing the synthesis of **130.**

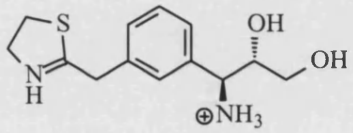
Confirmation of the ester exchange came from the loss of the methyl signal around δ 3.70 and the addition of two CH_2 peaks, an NH and an OH peak, while the FAB+ve ion mass spectrum showed the [M + H] peak at 427.

Deprotection of the Cbz and methyl ester was achieved using hydrogen bromide in acetic acid, cleaving the Cbz protecting group followed by hydrobromic acid, to cleave the methyl ester. Both targets **128** and **129** were isolated as the dihydrobromide salts. The most obvious way of deprotecting Cbz groups is with Pd/C, however, in this synthesis deprotecting Cbz with $Pd/C/H_2$ (in the final

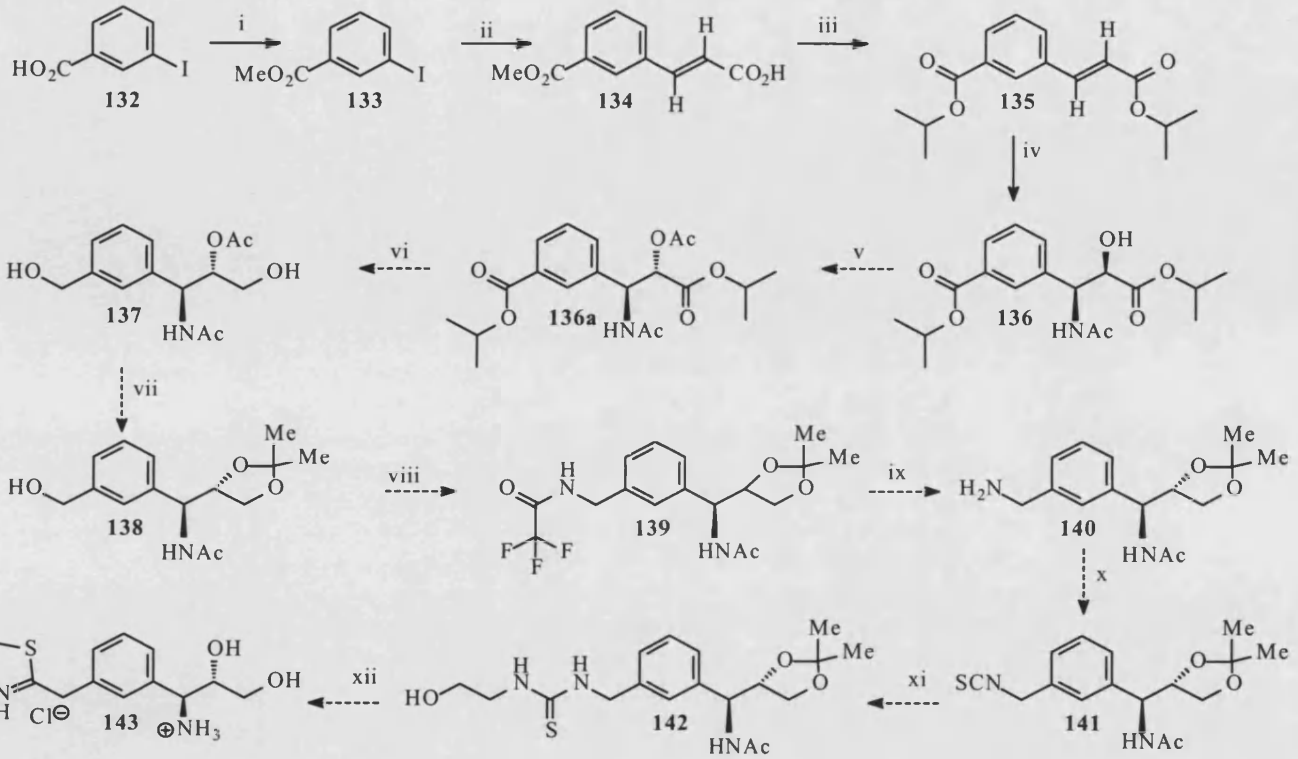
synthesis step) would have destroyed the head group as sulphur interferes with $Pd/C/H_2$. Another reason for deprotecting both protecting groups with hydrobromic acid is that the target would be isolated as the hydrobromide salt and not a mixture of different anions. In retrospect, the first starting material used (N^{ε}-Cbz-lysine); the Cbz protecting group could probably have been cleaved with hydrogen bromide in acetic acid instead of trying to remove the protecting group with $Pd/C/H_2$.

Synthesis of 2-(3-(1(S), 2(S)-1-Amino-2, 3-dihydroxypropyl) phenylmethyl-4,5-dihydrothiazole (143)

This target compound was designed to incorporate many of the features believed to be beneficial for the inhibition of iNOS. The amine-diol functionality has been shown to be particularly beneficial to the selectivity of iNOS inhibition. Hallinan *et al* 1998 showed that the dihydroxyethyl motif in **18** increased the selectivity for iNOS by 700 fold compared to **5**. This group also showed that the chirality of the inhibitor was important, as the diasteroisomer showed a decrease of four times the potency in NOS inhibition. The dihydroxyethyl motif together with the benzene ring and dihydrothiazole head group should give rise to a potent and selective iNOS inhibitor. Scheme 3.19 shows the entire synthetic route that would have been taken to target **143** had time permitted, several of these steps were achieved and these however, are discussed below.



143



Scheme 3.19: Synthesis of target **143**. Reagents and conditions: i)$SOCl_2$, MeOH, 4 d; ii) propenoic acid, palladium (II) acetate, Et_3N, propanenitrile, reflux 1 h; iii) propan-2-ol, conc H_2SO_4, reflux, 24 h; iv) $K_2[OsO_2(OH)_4]$, $LiOH.H_2O$, 2-methyl-2-propanol, $(DHQ)_2PHAL$, water, N-bromoacetamide, 4°C; v) PhP_3, DEAD, acetic acid; vi) $LiBH_4$, THF, Ar; vii) 2,2-dimethoxypropane, TSOH; viii) CF_3CONH_2, PhP_3, DEAD; ix) NH_3, MeOH; x) $CSCl_2$, $CHCl_3$, 16 h, xi) 2-aminoethanol, acetone, reflux; xii) aq.HCl, 6M, 40 h, reflux.

Retrosynthesis shows that the synthesis of **143** can be divided into three parts; i) the aromatic part, ii) the dihydrothiazole head group functionality and iii) the diol functionality. As mentioned before, the benzene ring was chosen to maintain the inhibitor in the correct conformation for binding to the active site. The different routes to synthesising the head group functionality were discussed in detail earlier. The addition-elimination reaction with 2-methylthio-4,5-dihydrothiazole at reflux (180°C for 4 h in the absence of solvent) was considered too harsh for this particular reaction. The pathway involving 1,2-dibromoethane was very low yielding and, as there would be many steps in the synthesis of **143**, this would not be the most efficient pathway to take. The most suitable method for synthesising the dihydrothiazole head group would be to make the isothiocyanate, then the 2-hydroxyethylthiourea and finally cyclise the open chain thiourea under reflux in aq. hydrochloric acid or trifluoroacetic acid. The most important factor when synthesising the diol functionality was to ensure correct stereochemistry throughout the synthesis.

There are several methods for introducing stereogenic centres into compounds. The reagents and reaction conditions play a vital role in determining whether the two possible enantiomers are formed in equal amounts. Allylic alcohols can undergo Sharpless asymmetric epoxidation to yield epoxy alcohols. The opening of the epoxy alcohols can be carried out using sodium azide and ammonium chloride. This yields the corresponding azido diols in good regioselectivity (>10:1). The azido group can then be reduced with sodium borohydride to give the amino diols. The reduction can be carried out in the presence of di-tert butyl dicarbonate to give the Boc protected amino diol (Padron *et al* 1999).

Bruncko *et al* 1997 adapted a method of the asymmetric dihydroxylation which allows olefins to be converted to non-racemic protected amino alcohols in a single step. This method uses cinchona alkaloids as the ligand. The Hofmann rearrangement is often a competing reaction of alkali metal salts of N-chlorocarboxamides. Using the N-bromo derivative and lowering the temperature to 4°C, this side reaction could be suppressed. Amines bind to OsO_4 *in situ* as chiral ligands, causing it to add asymmetrically. These ligands cause enantioselective addition as well as accelerating the rate of reaction giving regioselectivity values of

>20:1. By lowering the reaction temperature and selecting the appropriate ligand, enantiomeric purity can be increased. This method was considered the most efficient and effective way of producing an asymmetric aminohydroxylation, obtaining the product in a one step reaction compared to three steps with the epoxidation method. Another advantage for this method is the stoichiometric amounts of oxidant that are used which enables the work-up and purification to be simplified greatly.

Isopropyl cinnamates are the best precursors for the asymmetric aminohydroxylation reaction, using the method as described by Bruncko *et al* 1997. The easiest way of introducing the isopropyl ester into the target would be to carry out an ester exchange. As shown in scheme 3.19, a suitable starting material for the synthesis of target **143** would be an iodobenzene. The carboxylic acid could be protected with a methyl ester and the iodide would provide a suitable starting material for introducing the double bond to the target. Double bonds can be introduced into the target in several different ways:

i) Arylation of alkenes by organopalladium compounds: The Heck reaction
ii) The condensation of aromatic aldehydes with anhydrides: The Perkin reaction
iii) The condensation of aldehydes or ketones, usually not containing an α hydrogen: The Knoevenagel reaction

The most appropriate method for the synthesis of **143** is to use is the Heck reaction. The Heck reaction can be used on simple alkenes or alkenes with a variety of functional groups. This reaction is stereospecific, yielding products from *syn* addition followed by *syn* elimination. Under the umbrella of the Heck reaction there are four different methods in which an arylpalladium reagent can be generated. They are;

i) by treatment of an aryl bromide with palladium-triarylphosphine complex.
ii) by treatment of an aryl iodide with palladium acetate in the presence of a base.
iii) by treatment of an aryl mercury compound.

iv) by the reaction of an aromatic compound with palladium acetate or palladium metal and silver acetate in acetic acid.

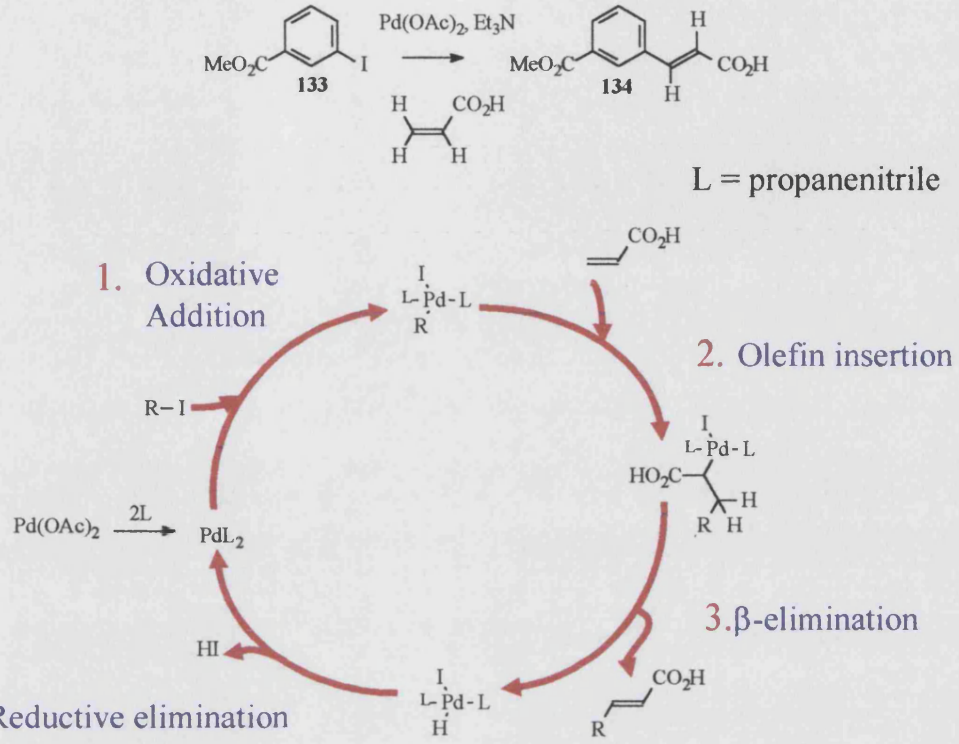
As mentioned above, the iodide would provide a suitable starting material for the Heck reaction. Previous members of our group developed an adaptation to the Heck reaction which could be carried out without the need for sealed tubes. The starting iodide would be heated with propenoic acid, palladium acetate and propanenitrile and refluxed for 1.5 h, with a straightforward work up (Watson 1997). This procedure is a far simpler way of introducing double bonds via the Heck reaction into target compounds.

Once the diol functionality had been synthesised, the next step would be to reduce the isopropyl esters with lithium borohydride to give the diol at one end of the compound and an alcohol at the other end. In order to change the remote alcohol function to an amine, the diol must be protected. This would be achieved by reaction with 2,2-dimethoxypropane to give an acetonide, as this only protects diols. A Mitsunobu reaction enables the replacement of a hydroxyl group by a wide range of nucleophiles (Hughes 1992), including trifluoroacetamide. The resulting amide can be cleaved to an amine group with ammonia. The dihydrothiazole head group is then able to be assembled in the way as described previously. The final cyclisation step in boiling aq. hydrochloric acid (6 M) would cleave the N-acetyl and acetonide protecting groups as well.

The order in which the synthesis is carried out is very important. The diol functionality was to be synthesised first. This would then be protected while the head group was synthesised. The final cyclisation step for the head group synthesis would be carried out in aq. hydrochloric acid as the acetyl protecting group would also be cleaved, leaving the desired target as the dihydrochloride salt.

As mentioned earlier, 3-iodobenzoic acid would be a suitable starting material. The first step in the synthesis involved protecting the carboxyl functionality with a methyl ester. This was achieved in high yield (98%) of crystalline solid. The next step required a Heck arylation reaction in which propenoic acid was coupled onto **133**. There are four steps in the Heck arylation reaction. These are; i) oxidative

addition, ii) olefin insertion, iii) β-elimination and iv) reductive elimination (scheme 3.20).



Scheme 3.20: The four steps in the Heck arylation reaction; i) oxidative addition ii) olefin insertion iii) β-elimination and iv) reductive elimination.

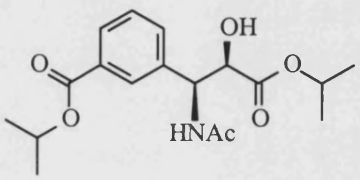
The propanenitrile acts as a ligand in this reaction and attaches to the palladium. During step one in the reaction, methyl 3-iodobenzoate (**133**) adds onto the palladium and ligand complex. Propenoic acid is inserted into the complex in step two and the product, methyl 3-(2-carboxyethenyl)benzoate (**134**) is β-eliminated in step three. Step four is a reductive elimination step in which hydrogen iodide is lost and the palladium ligand complex is recycled (scheme 3.20). The product **134** was achieved in quantitative crystalline yield and was isolated as the *trans* isomer. This was confirmed by the coupling constants between the CH-Ar and CH-CO_2H, J = 16 Hz.

The next step in the synthesis was to carry out an ester exchange on **134**. This step is important as the asymmetric hydroxylation requires bulky esters to give the reaction better regioselectivity. The procedure for the asymmetric hydroxylation (Bruncko *et*

al 1997) required isopropyl esters as the preceding step. The product was achieved in moderate yield (50%). The ^{1}H NMR clearly showed the disappearance of the methyl ester and the appearance of the doublets from by the propyl esters at δ 1.32 and 1.38.

Benzyl N-chlorocarbamate was the source of electrophilic nitrogen used in the asymmetric aminohydroxylation. This reagent would have to be synthesised as it is not commercially available. The advantage of using this compound would be that the amine functionality would be protected with a Cbz group which would easily cleave under the head group cyclisation conditions. The commercially available benzyl carbamate was monochlorinated by the procedure described by Bachand *et al* 1974. However, purification of the crude reaction mixture proved difficult and so an alternative was used instead. This was N-bromoacetamide which was commercially available and would leave an acetyl functionality protecting the diol. It was hoped the acetyl protecting group would be cleaved during the cyclisation of the dihydrothiazole head group (final step in the synthesis).

The asymmetric aminohydroxylation of **135** was carried out in a one-step reaction using the procedure as described by Buncko *et al* 1997. The olefin was converted to a non-racemic protected amino alcohol using osmium(VIII) and a cinchona alkaloid derivative ($(DHQ)_2PHAL$). The reaction was carried out at 4°C as this enhances the regioselectivity of the product. Compound **136** gave only one Rf value on TLC indicating that one diasteroisomer was present (Rf 0.4 (acetone/hexane 1:1)). Equal quantities of both enantiomers would give rise to $[\alpha]_D^{20}$ values of zero, and since **136** has an $[\alpha]_D^{20}$ value of -12.5° this signifies that there is a larger percentage of one of the enantiomers. Further analysis however, is required to establish whether **136** is in the pure enantiomeric form. Due to the method followed being a procedure for the *trans* addition it can be concluded that **136** is most likely to be the enantiomer shown below.



136

Most of the protons were readily assigned in the ^{1}H NMR spectrum of **136**. However, the assignment of the four CH peaks, the NH and the OH peak can not be done by ^{1}H NMR spectrum alone. It is most likely that the two multiplets at δ 5.10 and 5.24 correspond to the isopropyl CHs, while the doublet peak at δ 6.88 correlates to the NH peak. Assumptions are made in this assignment and so more evidence is needed to determine fully the structure of **136**. ^{1}H COSY and ^{1}H NOESY spectra were taken of **136**; these are given in figures 3.1 and 3.2, respectively. These spectra will help assign which peaks are correlated to each other and so assign fully the proton signals in the ^{1}H NMR spectra of **136**.

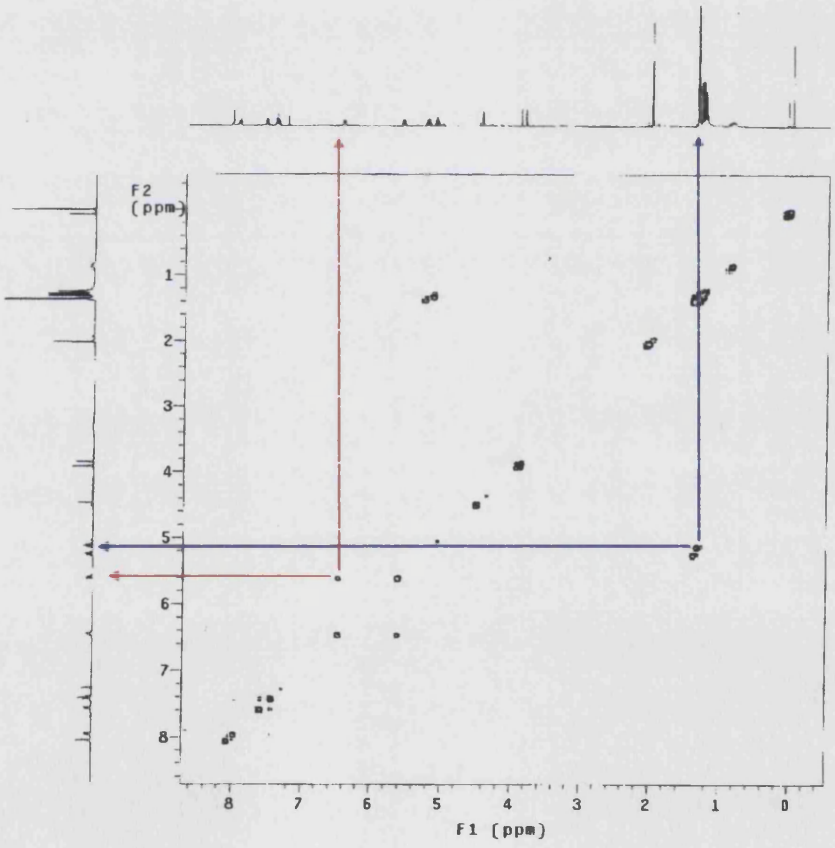


Figure 3.1: A ^{1}H COSY spectrum of compound **136**. The blue lines show the correlation between the propyl ester peaks and the corresponding CH peaks. The red lines show the correlation between the NH peak and the CH peak at δ 5.55.

The acetyl peak is seen at δ 1.97 on ^{1}H NMR. On the ^{1}H NOESY spectrum, through-space correlation is seen from this acetyl peak to a peak at δ 6.88. Since the acetyl is next to the NH, the peak at δ 6.88 must correspond to the NH. The ^{1}H COSY spectrum shows coupling from the NH peak to a peak at δ 5.55. The ^{1}H NOESY spectrum shows through-space correlation from the unassigned CH peak at δ 5.55 to the singlet at δ 4.46 and to two aromatic peaks at δ 7.56 and 8.06.

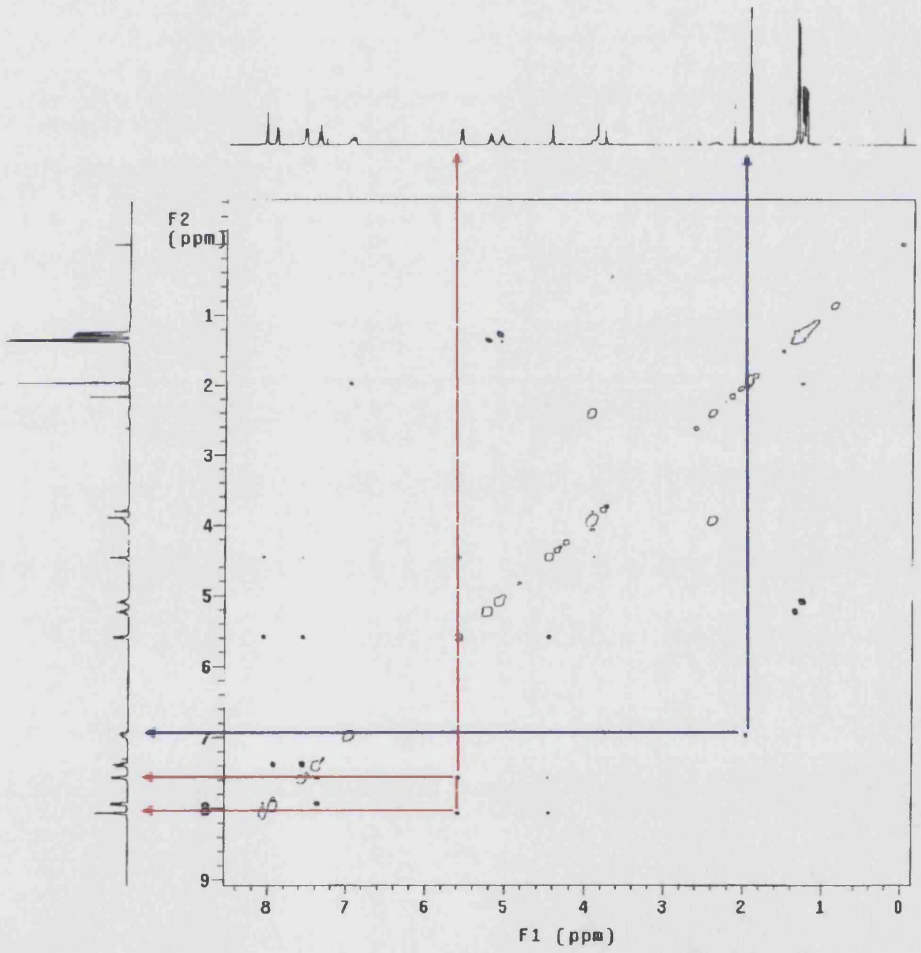


Figure 3.2: A NOESY spectrum of compound **136**. The blue lines show the correlation between the acetyl peak and the NH peak. The red lines show the correlation between the singlet at δ 5.55 to the aromatic protons at δ 7.56 and 8.06.

Therefore it is possible to conclude that the doublet peak seen at δ 5.55 must correspond to the C*H*NH. This is because through-space correlation is seen to the

two nearest aromatic peaks (Ar 2 and 6) the C*H*OH and also the acetyl peak. The broader singlet at δ 4.46 must correspond to the C*H*OH.

The ^{1}H COSY spectrum also shows that the two propyl ester peaks (δ 1.28 and 1.39) are coupled with the multiplet peaks at δ 5.10 and 5.24. This indicates that the multiplets must be CH peaks of the propyl esters.

The critical step in the synthesis of target **143** was the asymmetric hydroxylation, which was achieved in a reasonable yield (32%). Had time permitted, the remainder of the synthetic pathway as described above would have been carried out.

Synthetic routes to achieving the correct assignment of dihydrothiazole protons on ^{1}H NMR Spectrum

Introduction

The dihydrothiazole head group is a five-membered ring which possesses two CH_2 units, one adjacent to a nitrogen atom and the other next to a sulphur atom. Difficulties have arisen in analysing the ^{1}H NMR spectra of the dihydrothiazole head group functionality. The CH_2 units in the head group occur on the ^{1}H NMR spectra as two separate signals either as multiplets or triplets. However, due to the similar electronegativity properties of nitrogen and sulphur, it is unclear which of the CH_2 units resonates further downfield and which is more upfield. For example, in **123**, the two dihydrothiazole unit signals are seen as multiplets at δ 3.60 and 3.75. In another example (**67**), the two CH_2 signals are seen as triplets at δ 3.55 and 4.05. In both examples, it is unclear which signal corresponds to the CH_2 which is next to the nitrogen atom and which is the CH_2 adjacent to the sulphur. The aim of this study was to develop and synthesise compounds that would enable the two dihydrothiazole CH_2 units to be differentiated in such a way that would lead to the correct assignment of the two CH_2 signal peaks in the ^{1}H NMR spectra.

Several other advantages arise from achieving the aim of this study and they are;

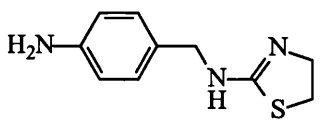
i) Evidence for the mechanism of ring closure. The most likely mechanism involves the OH group being protonated first and then the sulphur acting as a nucleophile and attacking the CH_2 adjacent to the OH_2^+ and so ring-closing the carbon chain in an S_N2 type reaction (scheme 3.3). However, S_N1-like mechanisms are also possible.

ii) Information gained from the study of deuterated dihydrothiazoles can provide a good base for labelled studies with ^{3}H or ^{14}C.

Results

A HETCOR spectrum enables the ^{13}C NMR spectrum to be correlated with ^{1}H NMR spectrum. Taking a HETCOR spectrum of one the dihydrothiazole target compounds would provide a very quick and simple way of assigning the CH_2 peaks on the ^{1}H NMR spectrum. Compound **75** was considered a good example for this analysis as the target was isolated as the free base and not as a hydrochloride or trifluoroacetate salt. This means that any effects that the salt may have on the spectra would be eliminated. The assignment of the peaks in the ^{13}C NMR spectrum of **75** showed that the CH_2S and CH_2N were a considerable distance apart (CH_2S at δ 35.7 and the CH_2N signal at δ 60.4) (Williams and Fleming 1980). A HETCOR spectrum was taken of this compound to see whether the CH_2S and CH_2N in the ^{13}C NMR spectrum could be correlated to the dihydrothiazole CH_2 triplets seen at δ 3.33 and 4.02 in the ^{1}H NMR spectrum.

The HETCOR spectra shows that the CH_2S signal in the ^{13}C NMR spectrum strongly correlates with the triplet at δ 3.33 while the CH_2N signal at δ 60.4 is linked with the triplet at δ 4.02 (figure 4.1). Although this information strongly suggests that the dihydrothiazole peaks are assigned with the CH_2N further downfield than the CH_2S assumptions are made about the correct assignment of the CH_2S and CH_2N peaks in the ^{13}C NMR spectrum. Therefore, more evidence is needed to confirm the assignment of the two dihydrothiazole peaks. The HETCOR spectrum provides no knowledge of the mechanism of cyclisation and so further synthetic routes were designed to try and establish the mechanism and the correct assignment.



Compound **75**

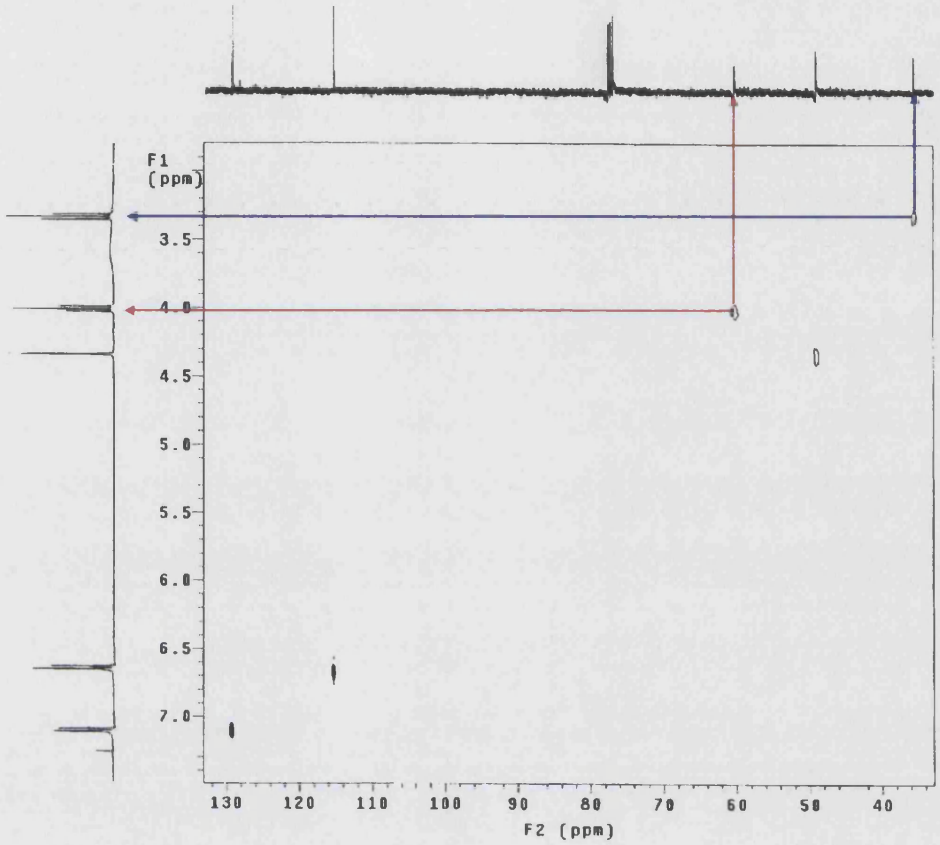
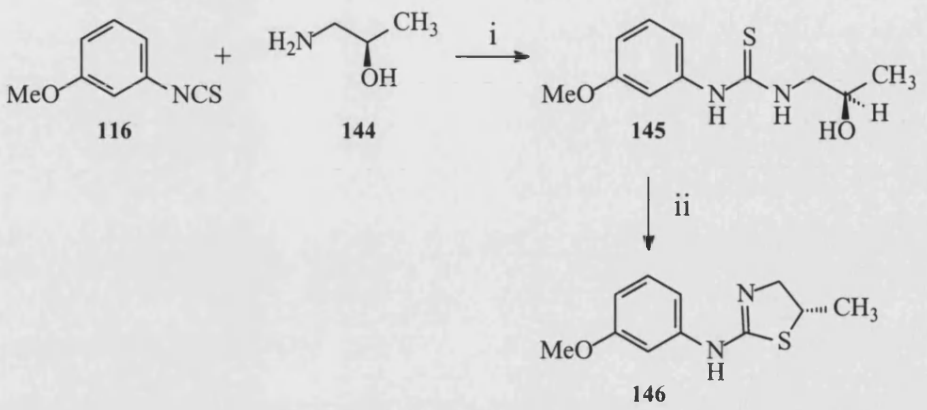


Figure 4.1: HETCOR spectrum of compound **75**. The blue line indicates the correlation between the CH_2S signal on the ^{13}C NMR spectrum and the triplet at δ 3.33 seen on the ^{1}H NMR spectrum. The red line indicates the correlation between the CH_2N signal at δ 60.4 on the ^{13}C NMR spectrum and the triplet at δ 4.02 seen on the ^{1}H NMR spectrum.

The first synthetic approach to achieving the aim of this project was to synthesise (S)-2-(3-methoxyphenylamino)-5-methyl-4,5-dihydrothiazole (**146**). The theory behind using 1-aminopropan-2-ol (**144**) was that the addition of the methyl unit into the dihydrothiazole head group would enable the dihydrothiazole CH_2 signals to be easily differentiated. The introduction of the methyl group into the dihydrothiazole head group would hopefully not alter the dihydrothiazole CH_2 signals too greatly from where they more commonly resonate. In addition, information could be gained

about the mechanism of cyclisation and ascertain whether, in fact, the cyclisation reaction proceeds by a S_N1 or S_N2 type reaction.



Scheme 4.1: Reagents and conditions: i) Acetone, reflux 2 h; ii) aq. HCl (6M), reflux 24 h.

The synthesis of **146** was straightforward and easy to accomplish as it only involved two steps (scheme 4.1). The addition of **144** to **116** was achieved in reasonable yield (38%) by boiling under reflux in acetone for 2 h. The ^{1}H NMR spectrum results showed that the CH_2 was no longer seen either as triplet or a multiplet peak but as two separate multiplet peaks (δ 3.64 and 3.94). This is due to the chiral centre that has been introduced. The chiral centre places both protons in the CH_2 unit in different magnetic environments and so each proton is visualised as a separate peak.

The cyclisation of **145** was carried out under reflux for 24 h in aq. hydrochloric acid (6 M), which gave the product **146** in a quantitative yield. Confirmation of the cyclised product came from the mass spectrometry results (FAB+ve ion 223 [M + H]). The ^{1}H NMR spectrum showed a multiplet at δ 3.77, corresponding to one proton and a multiplet at δ 4.12, accounting for two protons. It is unclear whether the protons in the CH_2 are seen separately as in **145** or together (as seen in all the other dihydrothiazole targets) at δ 4.12. A ^{1}H COSY spectrum enables the CH_2 and the CH of the dihydrothiazole to be correctly assigned (figure 4.2). The two protons from the CH_2 unit must be seen as two separate signals as coupling is observed from the signal at δ 4.12 to δ 3.77. If, however, the CH_2 unit was assigned as the multiplet

seen at δ 4.12, coupling would not be observed; the ^{1}H COSY spectrum shows that this is not the case. The evidence suggests that the CH_2S peak is further downfield from the CH_2N. This is a surprising result as the HETCOR spectra (figure 4.1) seen from compound **75** suggests that the CH_2N and CH_2S would appear on the ^{1}H NMR spectrum the other way around. It is most likely that the methyl unit introduced into the dihydrothiazole ring has a larger effect on the ^{1}H NMR spectrum than anticipated. Therefore, other synthetic routes were explored to establish the aim of the project.

The chiral centre within **146** provides evidence for the mechanism of cyclisation. The mechanism of ring closure is most likely to proceed by an S_N2 type reaction. The methyl group and hydroxyl group in the open chain thiourea increase the steric bulk of the compound, which pushes the mechanism towards an S_N1 type reaction. The S_N1 type reactions give rise to racemic products as the carbocation in step two of the mechanism can be attacked from either the top side, yielding one enantiomer or from the bottom side yielding the other enantiomer. When racemisation occurs, the $[\alpha]_D^{20}$ values of such compounds are zero. The mechanism of an S_N2 reaction of a pure enantiomer gives inversion of chirality. The nucleophile collides with the back side of the tetrahedral carbon (in this case the one with the hydroxyl and methyl group attached) causing the three groups attached to the carbon to flatten out into the transition state. The groups then flip over to the other side of the carbon giving rise to the opposite enantiomer as the product. The cyclised product **146** has an $[\alpha]_D^{20}$ value of -32.4° and, therefore, is it possible to conclude that the product has not racemised. Without further analysis it is unclear whether the reaction proceeds entirely by an S_N2 type mechanism or a mixture of S_N1 and S_N2. However, on the evidence given the reaction is most likely to proceed by an S_N2 type mechanism. Although the synthesis of 2-(3-methoxyphenylamino)-5-methyl-4,5-dihydrothiazole has given evidence for the mechanism of ring closure, the correct assignment of the protons in the unsubstituted dihydrothiazole remains to be finally resolved.

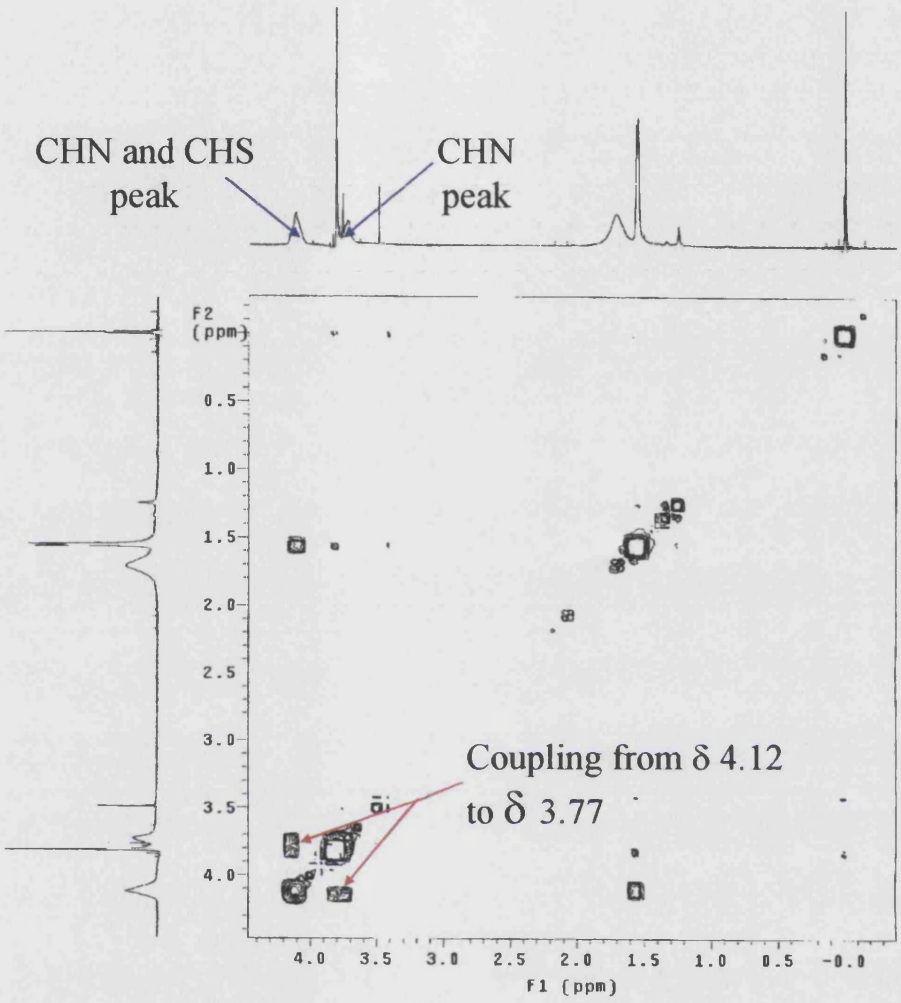
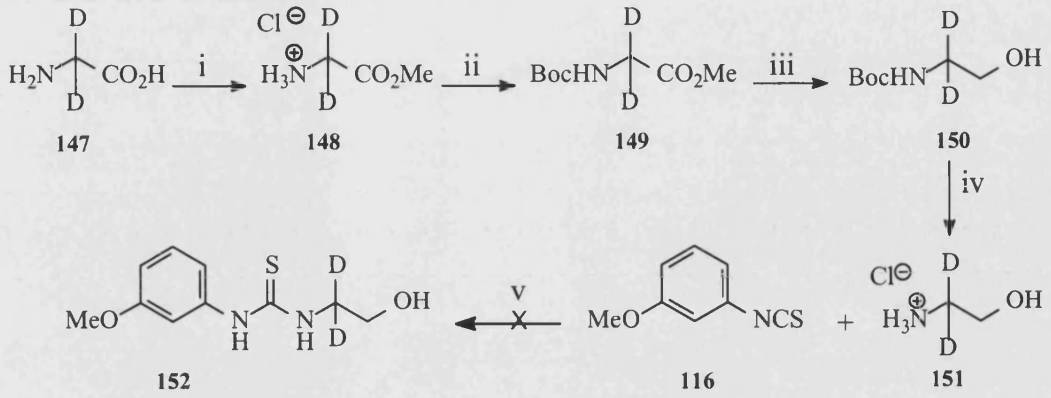


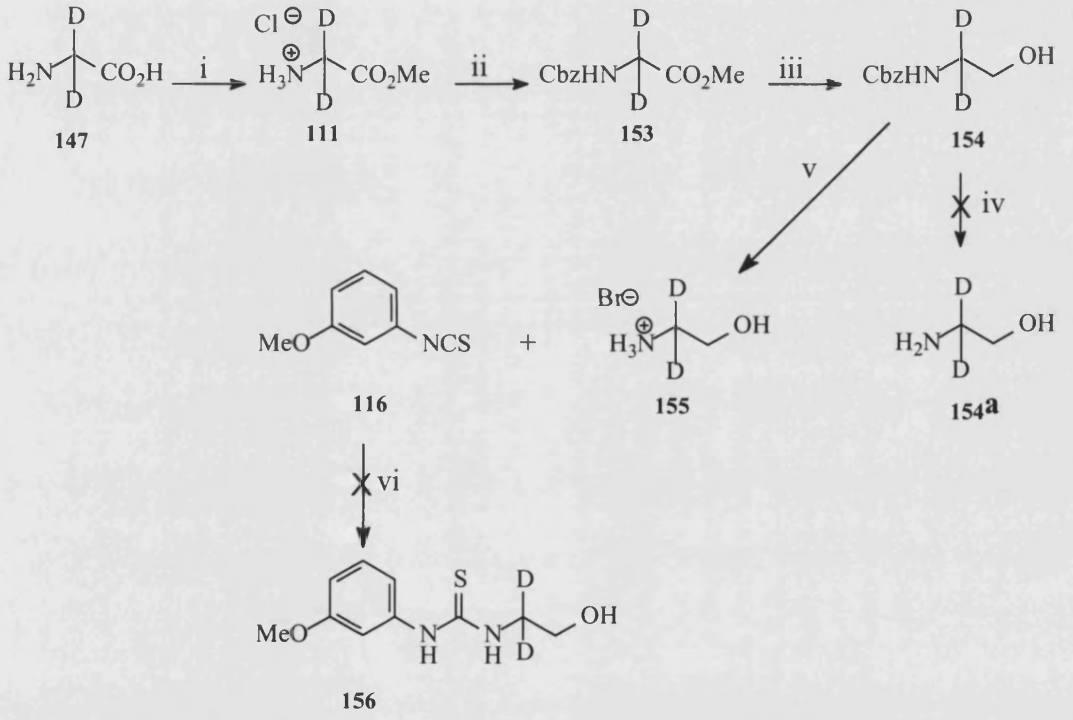
Figure 4.2: A ^{1}H COSY spectrum of compound **146**. The red line indicates the coupling observed from δ 4.12 to δ 3.77. The blue line shows the assignment of the CHN and CHS peaks.



Scheme 4.2: Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) Boc_2O, Et_3N, CH_2Cl_2, 16 h; iii) $LiBH_4$, THF, 16 h, Ar; iv) aq. HCl (6 M), 10 min; v) Acetone, reflux 2 h.

Another approach to the aim of this study was to synthesise a dihydrothiazole head group with one of the CH_2 units in the five-membered ring possessing a deuterium instead of hydrogen. Retrosynthesis showed that the simplest route to achieve a deuterated dihydrothiazole would be to make deuterated ethanolamine and add this onto a model isothiocyanate and cyclise the product in aq. hydrochloric acid following the same procedures for making dihydrothiazoles as before (scheme 4.2). The initial starting material used was α,α-dideuteroglycine (**147**) which was protected at the carboxyl terminal with a methyl ester. The product was obtained in a quantitative yield. The next step in the synthesis was to protect the amine; this was done using the most common amine protecting group, Boc. The reducing agent lithium borohydride was used to successfully reduce **149**, as it is selective for esters in the presence of carbamates. The Boc protecting group was removed with aq. hydrochloric acid (6M) from the product, yielding deuterated ethanolamine as the hydrochloride salt. Compound **151** was then treated with the model 3-methoxyphenylisothiocyanate (**116**) (which was chosen as it was commercially available and had no other reactive functionality). This reaction, after repeated attempts, proved unsuccessful. ^1H NMR spectra and mass spectra analysis showed no signs that the deuterated ethanolamine had been added onto the isothiocyanate. This may have been due to the deuterated ethanolamine hydrochloride salt being too

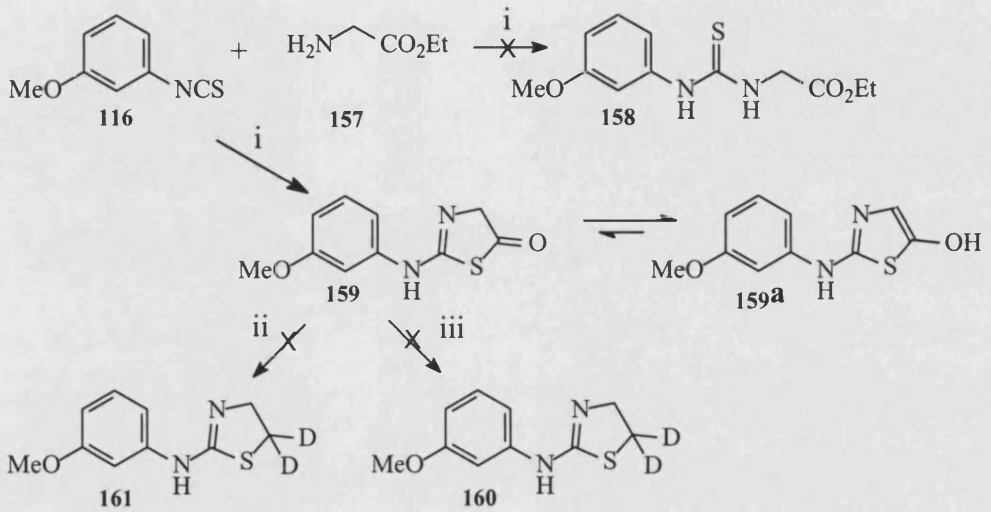
acidic to react with the isothiocyanate. Scheme 4.3 shows a similar synthesis but, instead of using Boc protecting group, Cbz was used. Cbz groups usually cleave easily with catalytic hydrogenation. However, this procedure gave no successful unprotected amine. The protecting group was removed with hydrogen bromide to give the deuterated ethanolamine hydrobromide. Once again, this was unsuccessful as the ^{1}H NMR and IR spectra showed that whatever was happening mechanistically in the addition of **151** with **116** was being mimicked in the addition of **155** with **116**. Clearly, a more basic approach was needed to couple the thiocyanate to the ethanolamine.



Scheme 4.3: Reagents and conditions: i) $SOCl_2$, MeOH, 4 d; ii) benzyl chloroformate, Et_3N, CH_2Cl_2, 16 h; iii) $LiBH_4$, THF, 16 h, Ar; iv) Pd/C H_2 16 h; v) HBr in HOAc (17.5%) 15 h; vi) Acetone, reflux 2 h.

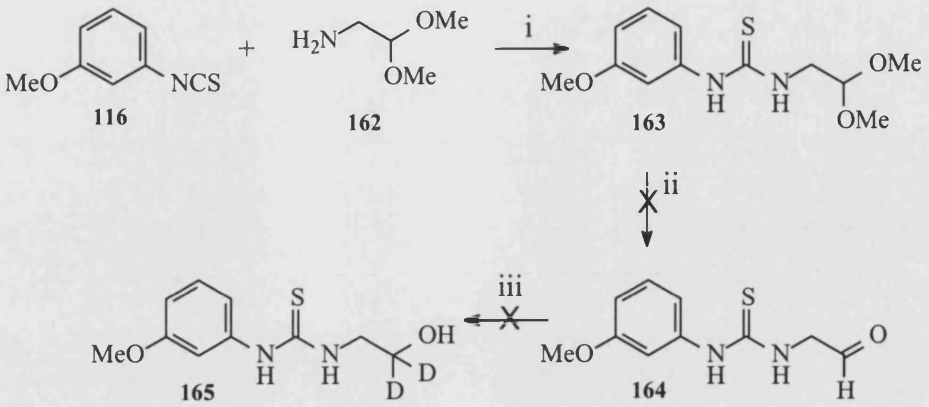
Attempts were made in which **148** was added directly onto **116** and then reduced. This time, pyridine was used as the solvent instead of acetone and also triethylamine was added in two equivalents in order to make the reacting mixture more basic.

These changes in the reaction conditions made no difference to the success of achieving the final product and so another approach was required.



Scheme 4.4: Reagents and conditions: i) Acetone, reflux 16 h; ii) $LiAlD_4$, THF, 16 h, Ar; iii) $NaBD_4$, EtOH, 16 h.

Scheme 4.4 shows the next synthetic route that was attempted. The idea was to use glycine ethyl ester (not deuterated) which would be added onto the model isothiocyanate and then reduced with a deueterated reducing agent, hopefully yielding the deuterated dihydrothiazole. The addition of glycine ethyl ester (**158**) produced not the desired product but gave the aromatic enol. Evidence for the cyclised product was confirmed by mass spectra (EI + 222 (M + H)). As scheme 4.4 shows failed attempts were made to reduce the enolate with either sodium boro-deuteride or lithium aluminium deuteride. Basic conditions favour the enolate form and so these compounds are not electrophilic enough for the reduction of the carbonyl to the CH_2. Once again another approach was needed.



Scheme 4.5: Reagents and conditions: i) Acetone, reflux 2 h; ii) Dowex 50×8 H^+, THF, reflux 16 h; iii) $NaBD_4$, acetone, 60°C, 16 h.

Scheme 4.5 shows another approach to achieving the deuterated dihydrothiazole using 2,2-diethoxyethylamine (**162**). The initial step in the synthesis involved the addition of **162** to the model isothiocyanate (**116**). This novel compound was achieved in reasonable yield (50%). The aim of the following steps in the synthesis was to synthesise the corresponding aldehyde which would then be reduced with a deuterated reducing agent such as sodium borodeuteride and finally cyclised to the deuterated dihydrothiazole. The most common method for obtaining aldehydes from acetals requires hydrolysis in aq. hydrochloric acid followed by a complicated work-up. This method was deemed unsuitable for the compounds involved in this synthesis. Another method for the hydrolysis of acetals is given by Webb and Threadgill (1990). This procedure involved a more gentle approach by boiling under reflux with Dowex 50×8 H^+ resin in wet THF followed by a simple filtration work up to remove the Dowex. This method was applied to **159**. Compound **159** was boiled under reflux with Dowex 50×8 H^+ to form the aldehyde and then reduced with $NaBD_4$. The reaction conditions were obviously too harsh for this compound as the ^1H NMR spectrum of the crude reaction mixture showed that the compound had decomposed.

Conclusions

Initially the synthesis of the methyldihydrothiazole **146** appeared to be a good strategy to assign the multiplets of the dihydrothiazole but the ^{1}H COSY spectrum suggests that the chiral centre introduced into the ring is believed to play a larger role than anticipated. The CH_2N was no longer seen as either a multiplet or a triplet but as two separate singlet signals. Evidence from the ^{1}H COSY spectrum of **146** and the HETCOR spectra of **75** are not consistent with each other. The ^{1}H COSY spectrum provides evidence that the CH_2N is further upfield of the CH_2S while the HETCOR spectrum suggests the opposite. Target deuterated dihydrothiazoles from other synthetic routes were not achieved. This means that there is insufficient evidence to back up the conclusions from the HETCOR or the ^{1}H COSY spectrum. However, it is most likely that the assignments of the two CH_2 peaks in the dihydrothiazole head group are consistent with the HETCOR spectrum, as the methyl group and the chiral centre introduced new variables into the project. Therefore, it is proposed, on the evidence given above, that the CH_2S peak resides further upfield to the CH_2N peak.

The synthesis of **146** did, however, provide evidence for the mechanism of ring closure for the dihydrothiazoles. The cyclisation of open-chain N-(2-hydroxyethyl)thioureas is believed to occur by an S_N2 type mechanism.

Evaluation of Target Compounds as Nitric Oxide Synthase (NOS) inhibitors

Introduction

There are several different methods used to determine NOS activity. Several techniques involve the measurement of the oxygenation products of NO. The Greiss reaction assays the nitrite ion, a chemical breakdown product of NO. NO interacts with naphthylethylenediamine (present in the Greiss reagent) in acid to produce a purple azo dye, which is measured at 548 nm on a uv/visible spectrophotometer. This method is particularly useful if the measurement of nitrate and nitrite is required (Ogden and Moore 1995). Thin-layer chromatography (TLC) exploits the fact that [^{3}H]-arginine and citrulline migrate different distances on the TLC plate, within a specified buffer system. The products can then be quantified either by radiometric scanning of the TLC plate or by scintillation counting (Kumar *et al* 1999). Other methods include chemiluminescence (Ogden and Moore 1995) and high performance liquid chromatography (HPLC) (Bredt and Snyder 1989). The major disadvantage with the methods described above occur when large sample numbers are involved, the assay then becomes time consuming and difficult to manage.

The assay involving the conversion of radiolabelled arginine to citrulline is probably the most widely used and has the advantages of being suitable for crude homogenates or purified enzyme. The assay can be used on multiple samples and is relatively inexpensive. However, the disadvantages are that arginine may be converted to citrulline by different enzymes e.g. arginase and so care must be taken when carrying out the assay.

The procedure measures the conversion of L-[*U*-^{14}C]-arginine to L-[*U*-^{14}C]-citrulline and NO (Ogden and Moore 1995). A simple ion exchange separation of the substrate and product is carried out by batch addition of the Dowex -50W ((200-400), 8% cross-linked, Na^+ form cation exchange resin (Collins *et al* 1998). The excess cationic L-arginine becomes bound to the resin whereas the labelled L-citrulline produced is zwitterionic and therefore does not bind to the resin and can be measured

by liquid scintillation counting (Salter *et al* 1991). This method was used in evaluating target compounds for NOS inhibition.

NOS can be subdivided into two main groups: constitutive NOS and inducible NOS. Constitutive NOS consists of eNOS and nNOS and is Ca^{2+}/CaM-dependent whereas inducible NOS (iNOS) is Ca^{2+}/CaM-independent. The target compounds synthesised were evaluated as inhibitors of both iNOS and cNOS. cNOS was obtained from rat brain and iNOS was obtained from rat lung, or HT1080 iNOS transfected cell line.

Cell homogenates have been found to be an adequate source of NOS when screening compounds for isoform-selective inhibitors. The rat brain is the main source of the cNOS isoform whereas rat lung remains the largest source of the iNOS isoform (Ogden and Moore 1995). One drawback of using NOS isoforms derived from animal species is the fact that they may have different physical and pharmacological characteristics to the human NOS enzymes.

Materials and Methods

Chemicals

Lipopolysaccharide (LPS), calmodulin, NADPH, calcium chloride, DL-dithiothreitol (DTT), bovine serum albumin (BSA), Dowex -50W ((200-400), 8% cross-linked, Na^+ form cation exchange resin, L-arginine, L-citrulline, L-valine and the known NOS inhibitor L-NMMA (**4**) were all obtained from Sigma Chemical Co. (Poole, Dorset, UK). L-[*U*-^{14}C]-Arginine hydrochloride was obtained from Amersham–Nycomed–Pharmacia (UK).

Methods

Animal treatments

The enzymes were kindly extracted by Dr Edwin Chinje (University of Manchester).

Enzyme induction in Rat tissues

Male Wistar rats (200 – 300 g), fed *ad libitum*, and were injected intraperitoneally with 4 mg Kg^{-1} trichloroacetic acid-extracted lipopolysaccharide (LPS). After 6 h, the rats were killed and the brain and lungs were removed and used directly or snap

frozen in liquid nitrogen for subsequent preparations for NO synthase activity measurements.

Preparation of NOS Rat Tissue

Either freshly excised or frozen rat brain or lung were homogenised (Ultra-Turax T25 homogeniser) in 4 volumes of ice-cold buffer containing HEPES (10 mM, pH 7.4), sucrose (320 mM), EDTA (0.1 mM), DL-dithiothreitol (0.5 mM), leupeptin (10 $\mu g\ ml^{-1}$) soybean trypsin inhibitor (10 $\mu g\ ml^{-1}$) and aprotinin (2 $\mu g\ ml^{-1}$), sonicated and then centrifuged using the TLA-100.3 Fixed Angle Rotor in Beckman TL-100 Tabletop Ultracentrifuge at 15000 rpm at 4°C for 30 minutes. The resultant pellet was discarded and the post-mitochondrial supernatant (cytosol + microsomes) was then treated with previously activated cation exchange resin (Dowex -50W ((200-400), 8% cross-linked, Na^+ form) to remove endogenous arginine. The supernatant was incubated with the resin for 5 minutes and centrifuged to settle the resin. This process was repeated twice after which the cytosol was assumed to be free of endogenous arginine. Several small aliquots in cryotubes were stored at -70°C until required.

Generation of iNOS-transfected clones

An optimised mammalian expression vector (pEFIRES-P, courtesy of Dr. S. Hobbs, CRC Centre for Cancer Therapeutics, ICR, London) was designed to express human iNOS (hiNOS) cDNA (courtesy of Prof. Ian Charles, University of London). Expression was linked with the selectable marker gene (*pac*) at the level of mRNA and antibiotic selection (puromycin) directly enforces expression of the cDNA. It was observed that this arrangement was effective at producing stable clones and because puromycin exerts selective pressure on the whole expression cassette, incremental doses led to generation of clones expressing increasing levels of enzyme activity. This vector has been used to transfect the human fibrosarcoma cell line, HT1080 and a series of iNOS-expressing clones were produced. To avoid loss of viability through the cytotoxic consequences of excessive NO production, clones were grown in the presence of a non-toxic dose of the NOS inhibitor, L-NNA (100 μM). Routinely, clones were grown for 48 hours in the absence of puromycin and the NOS inhibitor prior to extracting the iNOS enzyme.

Cells were grown to near confluence in T-175 cm^3 flasks and harvested by trypsinisation. Cells were then washed twice in cold phosphate buffered saline (PBS) and homogenised in five volumes of ice-cold buffer containing HEPES (10 mM, pH 7.4), sucrose (320 mM), EDTA (100 μM), dithiothreitol (50 μM), leupeptin (10 μg mL^{-1}), soybean trypsin inhibitor (10 μg mL^{-1}) and aprotinin (2 μg mL^{-1}). The preparations were then sonicated using an MSE Soniprep 150 for 3 x 5s at a nominal frequency of 23 KHz and an oscillation amplitude between 5 and 10 μm. Samples were placed in ice between each sonication. These suspensions were allowed to stand in ice for a further 10 min, and then centrifuged at 9000 x *g* for 15 min at 4°C. The resultant pellet was discarded and the post-mitochondrial supernatant (cytosol and microsomes) was treated with a strong cation exchange resin (Dowex -50W ((200-400), 8% cross-linked, Na^+ form) to remove endogenous arginine. The supernatant was incubated with the resin for 5 min and centrifuged at 10000 rpm for 5 min in order to pellet the resin. This process was repeated twice, after which the cytosol was treated as free of endogenous arginine.

NOS Assay

The NOS enzyme was prepared from rat brain and lung tissue using the protocol as described by Knowles *et al* 1990.

Protocol

50 μl of either cNOS/iNOS crude tissue extract (diluted 1:3 with HEPES buffer) was added to 10 ml plastic tubes containing 100 μl containing 20 mM HEPES buffer pH 7.4, L-valine (50 mM), NADPH (125 μM), L-citrulline (100 μM), L-arginine (10 μM) and L-[*U*-^{14}C]-arginine (50 μCiml^{-1}), 10 μM tetrahydropterin, calmodulin (400 U m^{-1}), DTT (375 mM), BSA (75 mg ml^{-1}) and $CaCl_2$ (0.25 mM). Samples were incubated at 37°C. The reaction was terminated by the addition of 1.5 ml 1:1 (v/v) H_2O/Dowex -50W ((200-400), 8% cross-linked, Na^+ form); exchange was allowed to take place while samples were incubated for 20 min at rt. H_2O (1.675 ml) was added and the resin was allowed to settle before 2 ml of supernatant was removed, to which 4 ml of scintillation liquid was added and examined for the presence of L-[*U*-^{14}C]-citrulline by liquid scintillation counting (Beckman, LS380).

The activity of the Ca^{2+}-dependent NOS was determined from the difference between the L-[U-^{14}C]citrulline generated from the control samples and samples containing 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA). The background activity was obtained by adding EGTA (1 mM) and NOS inhibitor **4** (1 mM). The Ca^{2+}-independent activity of the enzyme was determined by subtracting the background activity from the activity of samples containing 1 mM EGTA.

Pre-incubation

The known iNOS inhibitor 1400W (**10**) has been shown to be a slow tight binding inhibitor giving an optimum potency after a 10 min pre-incubation time (Garvey *et al* 1997). Most of the targets synthesised are analogues of **10** and, therefore, pre-incubation time may play an important role in the evaluation of the potency of the inhibitor. Compounds were initially screened at 100 μM, both with a 10 min pre-incubation and without.

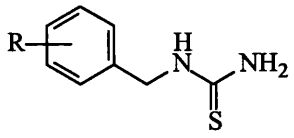
cNOS/iNOS preparation (40 μl) was added to 10 ml tubes containing the buffer mixture as above but without the arginine. The samples were pre-incubated at 37°C for pre-incubation time stated. Arginine mixture (10 μl) containing L-[*U*-^{14}C]-arginine (1 μl/tube) and arginine stock 10 mM was added. The samples were then incubated for 10 min and the procedure was conducted as before.

IC_{50} determinations

All target compounds were initially screened for their NOS inhibition at 100 μM concentration. The compounds which showed good inhibition (>70%) were assayed further to determine their IC_{50} values.

Each data point is expressed as a mean ± sd mean of duplicate determinations per experiment, unless otherwise stated.

Results and Discussion



R	Compound Number	% inhibition iNOS T=0	iNOS T=10	cNOS T=0	cNOS T=10
3-CH_2NH_2	**35**	34 ± 0.6	44 ± 2	39 ± 5	47 ± 0.1
4-CH_2NH_2	**40**	29 ± 5	32 ± 4	11 ± 6	20 ± 2
3-NH_2	**64**	-58 ± 1	9 ± 0.5	-1 ± 6	9 ± 6
4-NH_2	**71**	-55 ± 11	12 ± 0.7	-0.3 ± 1	12 ± 2
3-OMe	**110**	35 ± 2	7 ± 3	-3 ± 1	6 ± 4
4-OMe	**114**	34 ± 13	2 ± 5	8 ± 1	14 ± 0.2
3-CO_2H	**96**	-46 ± 5	7 ± 2	14 ± 0.6	-0.4 ± 2
4-CO_2H	**104**	-56 ± 12	3 ± 0.5	-0.4 ± 0.4	6 ± 0.4

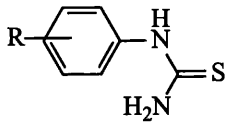
Table 5.1: % Inhibition results for thioureas against rat brain cNOS and hiNOS screened at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

Table 5.1 shows that pre-incubation time plays an important role in determining the optimum potency of the inhibitor. The most significant changes in NOS inhibition are seen in compounds **64, 71, 96** and **104**; these compounds showed high stimulatory values for iNOS at time zero. After a ten-minute pre-incubation with the enzyme these compounds changed from exhibiting stimulatory properties to being weak inhibitors. These compounds may be acting at a site, which is distant from the L-arginine binding site of the NOS enzymes and may be somehow increasing the catalytic activity of the enzyme.

Although **64, 71, 110** and **104** show stimulation with cNOS at time zero the percentage stimulation values are very low, indicating that they are more likely to be very weak inhibitors of cNOS rather than stimulators.

The two methoxy targets **110** and **114** showed a decrease in percentage inhibition with time for iNOS. Neither of these compounds showed any significant potency for either isoform.

A surprising result in this series of targets was that **35** and **40** were not as good inhibitors of iNOS and cNOS as previously predicted. Compound **40** was marginally better at inhibiting iNOS than **35** which is consistent with the results from Shearer *et al* (1997) that the para position around the aromatic ring gives rise to the most selective iNOS inhibitors. Compound **40** showed no significant increase in potency with time indicating that they are probably not slow-onset binding inhibitors.



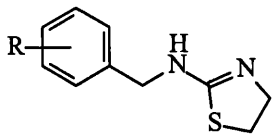
		% inhibition			
	Compound	iNOS		cNOS	
R	Number	T=0	T=10	T=0	T=10
3-CH_2NH_2	**48**	35 ± 4	40 ± 5	98 ± 3	67 ± 0.1
4-CH_2NH_2	**56**	57 ± 0.7	52 ± 0.5	48 ± 4	44 ± 1
3-OMe	**117**	17 ± 6	2 ± 0.6	-4 ± 1	5 ± 0.1
4-OMe	**121**	26 ± 7	28 ± 1	-1 ± 3	4 ± 2
3-CH_2CO_2H	**80**	-65 ± 5	-3 ± 14	9 ± 6	5 ± 2
4-CH_2CO_2H	**87**	15 ± 2	13 ± 3	-7 ± 8	15 ± 6

Table 5.2: % Inhibition results for thioureas against rat brain cNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation time the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

The removal of the methylene unit from between the aromatic ring and thiourea head group increased the potency of inhibitors, compared to those in table 5.1. The para-substituted CH_2NH_2 functionality (**56**) showed no time-dependence as seen in **40**. However, the meta-substituted CH_2NH_2 functionality (**48**) showed time-dependence towards the cNOS isoform but not the iNOS isoform. This was also seen in the homologous compound **35**. Compound **48** was clearly the most potent inhibitor in this group of thioureas also displaying a degree of selectivity for the cNOS isoform.

As seen in the previous class of thiourea targets, the methoxy compounds showed no significant inhibition of either isoform.

A high stimulation percentage was seen for iNOS for compound **80** at time zero. The CH_2CO_2H group appears to give rise to stimulatory compounds rather than NOS inhibitors, particularly when the aromatic ring was substituted in the meta position.



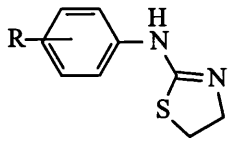
		% inhibition			
	Compound	iNOS		cNOS	
R	Number	T=0	T=10	T=0	T=10
3-CH_2NH_2	**31**	48 ± 0.1	26 ± 3	55 ± 6	59 ± 0.6
4-CH_2NH_2	**42**	-30 ± 2	51 ± 2	33 ± 3	65 ± 2
3-NH_2	**68**	-36 ± 0.5	12 ± 2	5 ± 2	17 ± 5
4-NH_2	**75**	-47 ± 10	19 ± 2	8 ± 2	12 ± 1
3-OMe	**112**	34 ± 4	8 ± 4	8 ± 1	14 ± 0.2
4-OMe	**21**	26 ± 6	3 ± 13	10 ± 2	13 ± 0.1
3-CO_2H	**99**	-39 ± 1	34 ± 3	5 ± 3	13 ± 3
4-CO_2H	**107**	-45 ± 0.1	5 ± 0.4	0.4 ± 4	-11 ± 0.1

Table 5.3: % Inhibition results for dihydrothiazoles against rat brain cNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

Compound **42** was the most potent inhibitor in this group of compounds showing reasonable potency but no selectivity for either isoform. The dihydrothiazole head group with the extra methylene unit is clearly more potent for both isoforms than the corresponding thiourea, as displayed by **42** and **40**, respectively.

Dihydrothiazoles substituted with the amine and carboxylate functionality in both the meta and para positions gave rise to stimulatory compounds at time zero with iNOS. The same functional groups gave stimulatory values for the corresponding thioureas (table 5.1).

Targets **112** and **21** showed a decrease in potency with time for the iNOS isoform and an increase in potency with time for the cNOS isoform. These results were also seen in the corresponding methoxyaryl thiourea targets **110** and **114** (table 5.1).



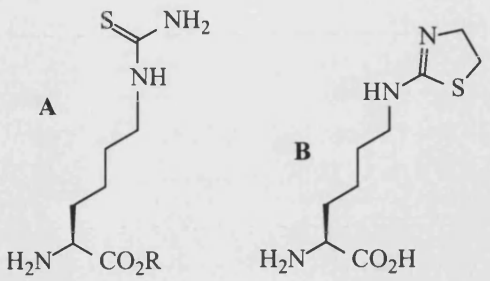
		% inhibition			
	Compound	**iNOS**		**cNOS**	
R	**Number**	**T=0**	**T=10**	**T=0**	**T=10**
3-CH_2NH_2	**50**	62 ± 2	86 ± 1	66 ± 0.6	89 ± 0.2
4-CH_2NH_2	**58**	16 ± 4	40 ± 0.7	56 ± 0.6	56 ± 1
3-OMe	**119**	26 ± 2	-1 ± 12	7 ± 0.3	20 ± 2
4-OMe	**123**	1 ± 0.4	7 ± 3	-2 ± 1	17 ± 0.1
3-CH_2CO_2H	**82**	-1 ± 4	-8 ± 14	4 ± 2	9 ± 3
4-CH_2CO_2H	**89**	-58 ± 4	9 ± 2	9 ± 2	14 ± 5

Table 5.4: % Inhibition results for dihydrothiazoles against rat brain cNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

Comparison of **31** and **50** show that the removal of the methylene unit from the dihydrothiazole target dramatically increases the potency of the inhibitor, especially for iNOS inhibition at the pre-incubation time of ten minutes (Compound **31** 26%, **50** 86%). Although the potency of the inhibitor was increased, no isoform selectivity was seen in **50** or **58** (the second most potent target in this series).

Stimulatory values are seen once again with the carboxyl functionality, with the highest stimulatory values seen when the target is substituted in the para position, with the iNOS isoform at a pre-incubation time of zero.

As seen in previous tables, the methoxy substituted targets showed no potency or selectivity for either isoform.



Type	Compound Number	R	% inhibition			
			iNOS		cNOS	
			T=0	T=10	T=0	T=10
A	**128**	Me	77 ± 5	67 ± 0.1	14 ± 0.4	90 ± 2
A	**129**	H	98 ± 0.3	98 ± 1	98 ± 2	99 ± 3
B	**131**	H	64 ± 0.1	37 ± 2	34 ± 2	12 ± 2

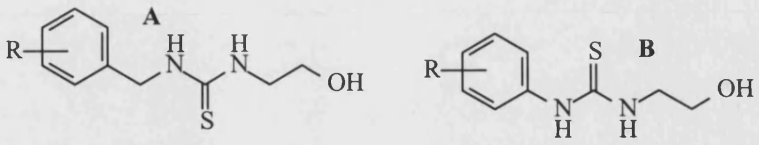
Table 5.5: % Inhibition results for lysine derivatives against rat brain cNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

The lysine derivatives showed a high potency for both isoforms. Compound **129** was the most potent but showed no time-dependent kinetics, whereas **128** showed a large increase in potency with time with the cNOS isoform.

Compound **131** was most selective for iNOS but, unlike most of the other targets tested this compound showed a reduction in potency with time with both cNOS and iNOS indicating that perhaps this compound is not a slow binding inhibitor.

Target **130** showed a decline in percentage inhibition when compared with the corresponding ornithine target **17**. Adding the extra CH_2 unit has a dramatic effect on potency of the inhibitor. Compound **17** gave IC_{50} values of 8.1 and 1.3 μM for iNOS and cNOS respectively (Ulhaq *et al* 1999), whereas **130** gave percentage inhibition values no greater than 64% at 100 μM for either isoform. It would appear that the shorter amino acid chain length gives rise to more potent inhibitors;

however, **128** and **129** show that this is not necessarily the case. These two targets gave high percentage inhibition values indicating that the head group plays a vital role in binding to the active site and determining the potency of the inhibitor.



Type	Compound Number	R	% inhibition			
			iNOS		cNOS	
			T=0	T=10	T=0	T=10
A	**111**	3-OMe	-58 ± 7	8 ± 0.3	-1 ± 4	5 ± 1
A	**115**	4-OMe	-25 ± 1	44 ± 5	-1 ± 1	5 ± 1
B	**118**	3-OMe	-10 ± 1	-1 ± 1	0.03 ± 0.6	1 ± 0.8
B	**122**	4-OMe	6 ± 3	6 ± 2	2 ± 1	2 ± 3

Table 5.6: % Inhibition results for 2-hydroxyethyl thioureas against rat brain cNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme. T=10 refers to a ten-minute pre-incubation time of the compound with the enzyme.

The methoxyisothiourea targets **110, 117, 114, 121** showed very little potency for either iNOS or cNOS with **110, 117** and **121** showing negative values, indicating that the activity was being stimulated rather than inhibited. Targets **111, 115, 118, 122** were synthesised to explore the SAR within the thiourea targets. The effects of chain length on the thiourea head group could then be discussed and determined whether this structural difference caused an inhibitory or stimulatory effect.

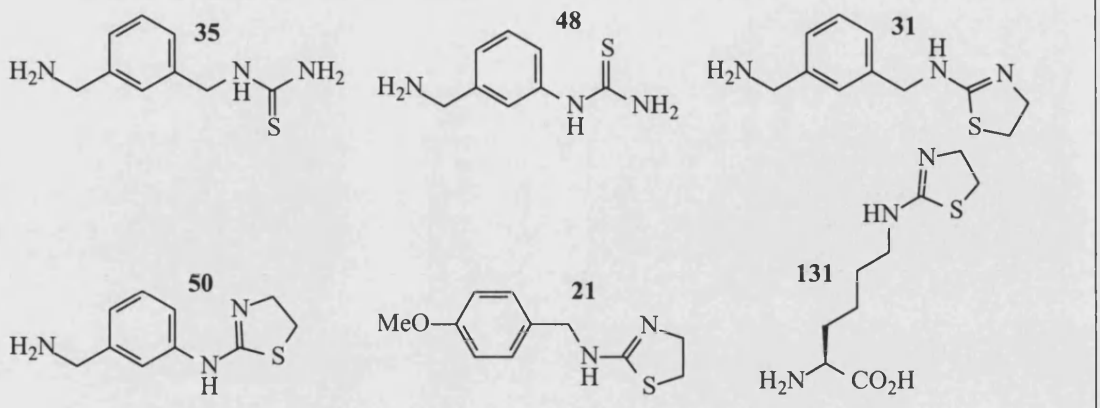
Targets which had the extra methylene unit between the head group and the aromatic ring (**111** and **115**) showed higher stimulatory values than those which had the head group directly attached to the ring (**118** and **122**), with the greatest stimulatory value seen in target **111**.

Introducing the 2-hydroxyethyl motif to the methoxy targets switches the values from inhibitory to stimulatory. This is illustrated by comparing targets **110** and **114** (table 5.1) with targets **111** and **115**. The meta-substituted targets switch from 35%

(**110**) inhibition, to 58% (**111**) stimulation, while the para-substituted targets switch from 34% (**114**) inhibition to 25% (**115**) stimulation. The same comparison can be made between the targets with the thiourea directly attached to the ring: **117** and **121** (table 5.2) with **118** and **122**.

The stimulatory effects are only seen in targets when screened against the iNOS isoform and not cNOS. The large stimulatory effects seen in this group of targets indicates that there are perhaps two separate binding sites; one for stimulation and another for inhibition. Had time permitted, it would have been interesting to test both the carboxyl and amine targets with the 2-hydroxyethyl motif.

Comparison of different sources of the iNOS isoform

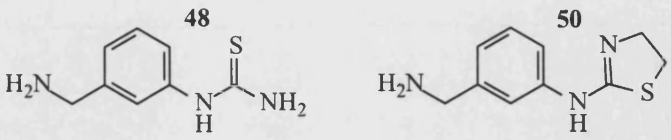


Compound Number	% inhibition iNOS (rat) T=0	% inhibition hiNOS T=0
35	23 ± 2	34 ± 0.6
48	98 ± 0.5	35 ± 4
21	5 ± 3	26 ± 6
31	34 ± 0.4	48 ± 0.1
50	34 ± 0.6	62 ± 2
131	21 ± 0.3	64 ± 0.1

Table 5.7: % Inhibition results for against rat lung iNOS and hiNOS at 100 μM. Values are expressed as mean ± SEM (n=2). T=0 refers to no pre-incubation of the compound with the enzyme.

Initial studies of some target compounds were carried out using rat iNOS and rat cNOS with no pre-incubation. These results are given in table 5.7.

The majority of studies on NOS inhibition have been carried out in rodent iNOS. Table 5.7 shows a comparison between human and rat sources of iNOS. Most of the six target compounds showed an increase in percentage inhibition with the human source of iNOS, only compound **48** showed a decrease in percentage inhibition with the human source.



Compound Number	IC_{50} (μM)		
	iNOS	cNOS	Selectivity (cNOS/iNOS)
50	23 ± 2	10 ± 0.8	2
48	190 ± 2	21 ± 1	9

Table 5.8: Comparison of IC_{50} values of compounds **48** and **50** against rat lung iNOS and rat brain cNOS. Values are expressed as mean ± SEM (n=3). IC_{50} values taken without a pre-incubation time.

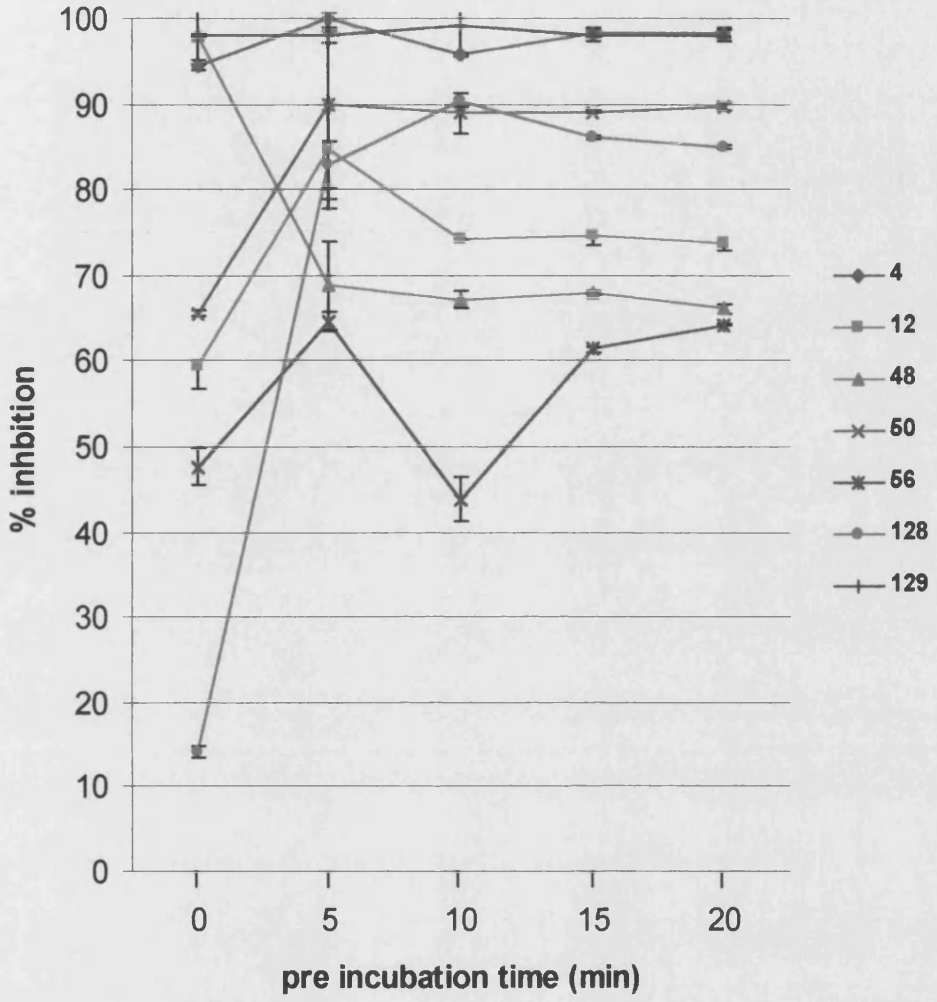
Compound **48** showed a nine-fold selectivity for the cNOS isoform over the rat iNOS isoform, while **50** only showed a two-fold selectivity. Table 5.11 shows that the selectivity displayed by these targets decreases when the human isoforms are used. Compound **48** shows only a five-fold selectivity, while **50** shows no selectivity for either human NOS isoform. Although **50** gives a more potent IC_{50} with the human source the selectivity is lost, whereas **48** displays more potent IC_{50} with the rat isoform (table 5.11).

This shows that each potential NOS inhibitor should be studied individually as their kinetics may be very different from each other.

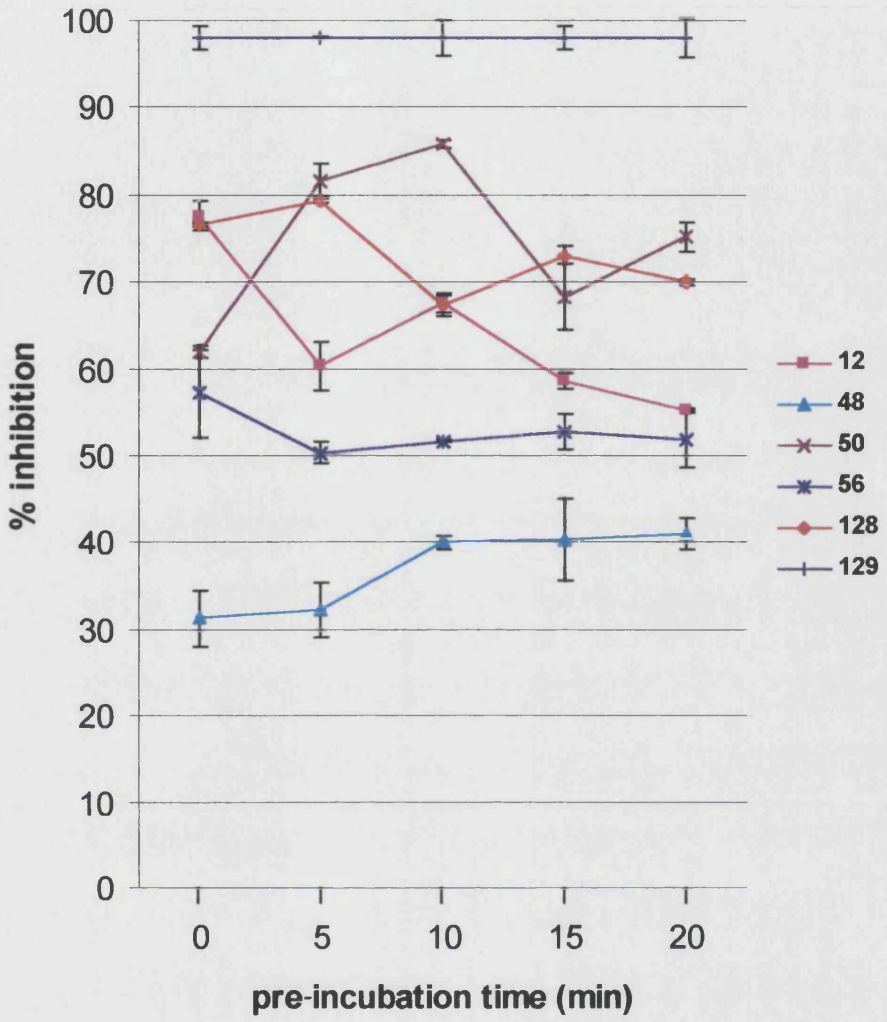
Time Study

The preliminary studies showed that five compounds gave high percentage inhibition values at 100 μM. These compounds (**48, 50, 56, 128** and **129**) were analysed further for IC_{50} results. Pre-incubating the target with the enzyme affects the potency of the inhibitor. To ensure the targets were assayed at their optimum pre-incubation time, time study curves were carried out in which the enzyme was pre-incubated with target at various time intervals. The five targets mentioned above were assayed with known inhibitors and against both cNOS and hiNOS isoforms.

As graphs 5.1 and 5.2 show pre-incubation time plays a large role in the potency of the inhibitor. The absence of pre-incubation time can lead to an under estimation of the potency of a slow-onset binding inhibitor. Exposure *in vivo* or in the clinical environment may be >30 min (Alderton *et al* 2001), so it is important to study time dependence in order to predict potency and selectivity of the inhibitor.



Graph 5.1: % Inhibition results for compounds **48, 50, 56, 128, 129** and known inhibitors **4** and **12** against rat brain cNOS at 100 μM, with varying times of pre-incubation (minutes) of the compound with the enzyme. Values are expressed as mean ± SEM (n=2).



Graph 5.2: % Inhibition results for compounds **48, 50, 56, 128, 129** and known inhibitor **12** against hiNOS at 100 μM, with varying times of pre-incubation (minutes) of the compound with the enzyme. Values are expressed as mean ± SEM (n=2).

Results and Conclusions

The optimum pre-incubation time chosen to assay targets with the hiNOS enzyme was fifteen minutes. Compounds **50** and **128** showed large increases in percentage inhibition after five minutes, while **48** showed a decline in percentage inhibition after five minutes. Compound **129** showed percentage inhibition values around 98% for all time points and, with hindsight, should have been assayed again at a lower

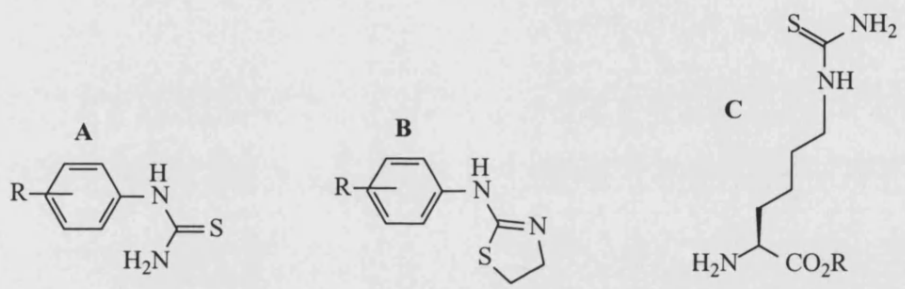
concentration to see if time-dependence was evident. Compound **56** showed a decline in percentage inhibition after ten minutes; this result was repeated several times to eliminate any human or assay errors.

The optimum pre-incubation time chosen to assay targets with the cNOS isoform was fifteen minutes. Compound **128** showed similar high percentage inhibition results with cNOS and was seen with iNOS. As mentioned before, this compound should ideally have been assayed again at a lower concentration to see if time-dependence was evident.

Clearly time plays an important role in determining the optimum potency of the inhibitor and also gives an insight into whether the inhibitors display time-dependent kinetics. Ideally, each target should be assayed individually at their corresponding optimum time point; however, this may lead to difficulties in comparing the results for different analogues appropriately.

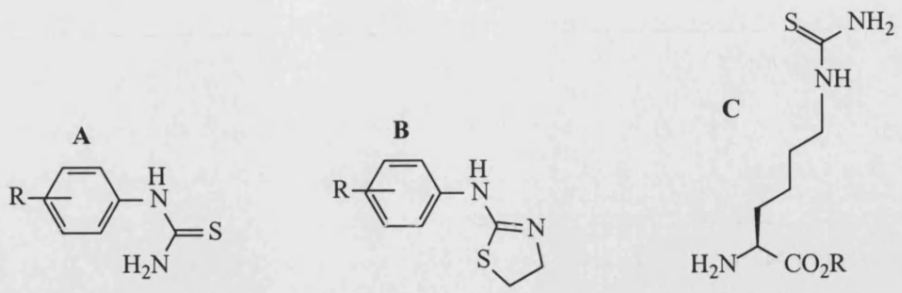
Arginine Concentration Study

The concentration of arginine used in published NOS assays varies immensely from 0.2-10 μM (Garvey *et al* 1994), 10 μM (Ulhaq *et al* 1998), 30 μM (Rees *et al* 1990) and 50 μM (Sorrenti *et al* 2001). Therefore, to determine whether arginine concentration plays a significant role in the potency of the inhibitor and whether 10 μM arginine gives the optimum percentage inhibition, a study was undertaken using the five most potent inhibitors as before and two known inhibitors.



Compound Number	Type	R	Arginine concentration (% inhibition) 10 μM	5 μM	3 μM
48	A	3-CH_2NH_2	68 ± 1	71 ± 1	73 ± 2
56	A	4-CH_2NH_2	61 ± 0.2	62 ± 0.8	66 ± 3
50	B	3-CH_2NH_2	89 ± 0.6	93 ± 3	91 ± 0.6
128	C	Me	86 ± 0.1	91 ± 2	89 ± 2
129	C	H	98 ±.04	98 ± 0.5	98 ± 0.4
4	-	-	98 ± 0.2	98 ± 0.1	97 ± 1

Table 5.9: % Inhibition results for compounds **48, 50, 56, 128, 129** and known inhibitor **4** against rat brain cNOS at 100 μM, at pre-incubation of fifteen minutes with varying arginine concentrations. Values are expressed as mean ± SEM (n=2).



Compound Number	Type	R	Arginine concentration (% inhibition) 10 μM	5 μM	3 μM
48	A	3-CH_2NH_2	40 ± 5	41 ± 4	61 ± 9
56	A	4-CH_2NH_2	53 ± 4	51 ± 1	50 ± 2
50	B	3-CH_2NH_2	85 ± 3	77 ± 7	74 ± 7
128	C	Me	73 ± 2	93 ± 0.1	97 ± 3
129	C	H	98 ± 1	98 ± 3	98 ± 0.1
10	-	-	98 ± 1	-	94 ± 0.5

Table 5.10: % Inhibition results for compounds **48, 50, 56, 128, 129** and known inhibitor **10** against hiNOS at 100 μM, at pre-incubation time of fifteen minutes with varying arginine concentrations. Values are expressed as mean ± SEM (n=2).

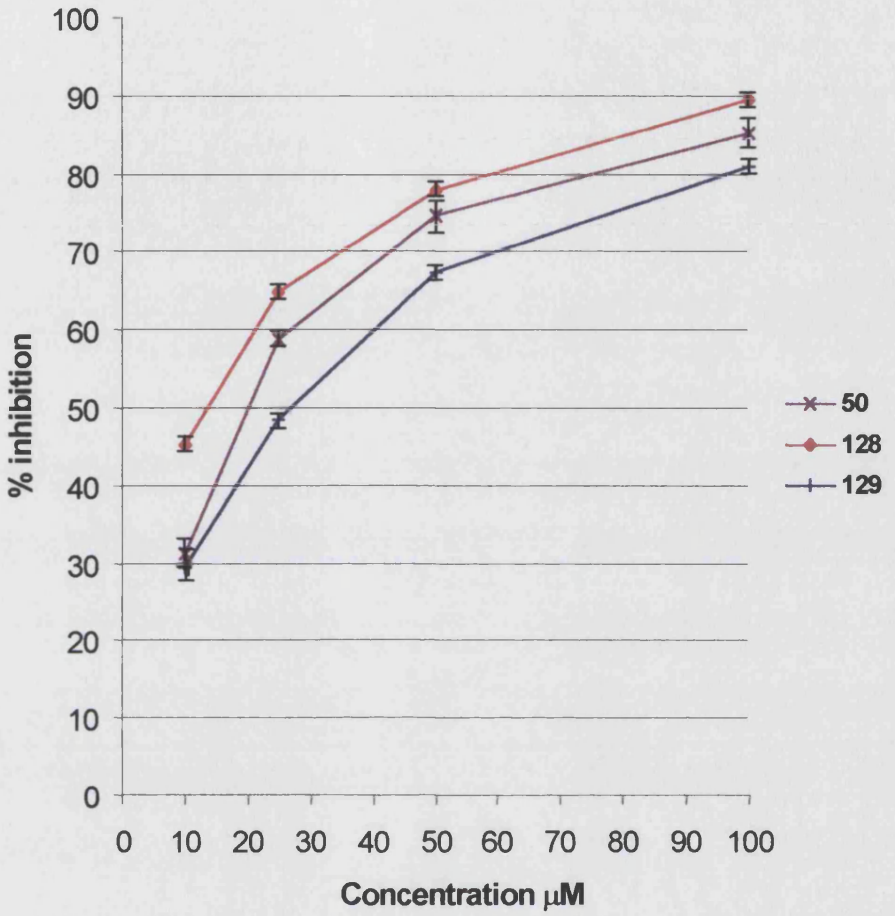
Results and Conclusions

Table 5.9 shows that compounds **48, 50, 56** and **128** display no significant difference in percentage inhibition with different arginine concentrations. Compound **129** shows percentage inhibition values >97% for all three arginine concentrations and was probably not assayed at the optimum concentration for that particular compound in this type of study. This is illustrated again in table 5.10 with **128** and **129**. Compound **50** is the only one that shows any significant increase in percentage inhibition with decreasing arginine concentrations. It would seem that each compound should be assayed at their own optimum concentration to achieve the best results. However, this would lead to incompatibility within the data and each target would not be able to be compared with any of the others.

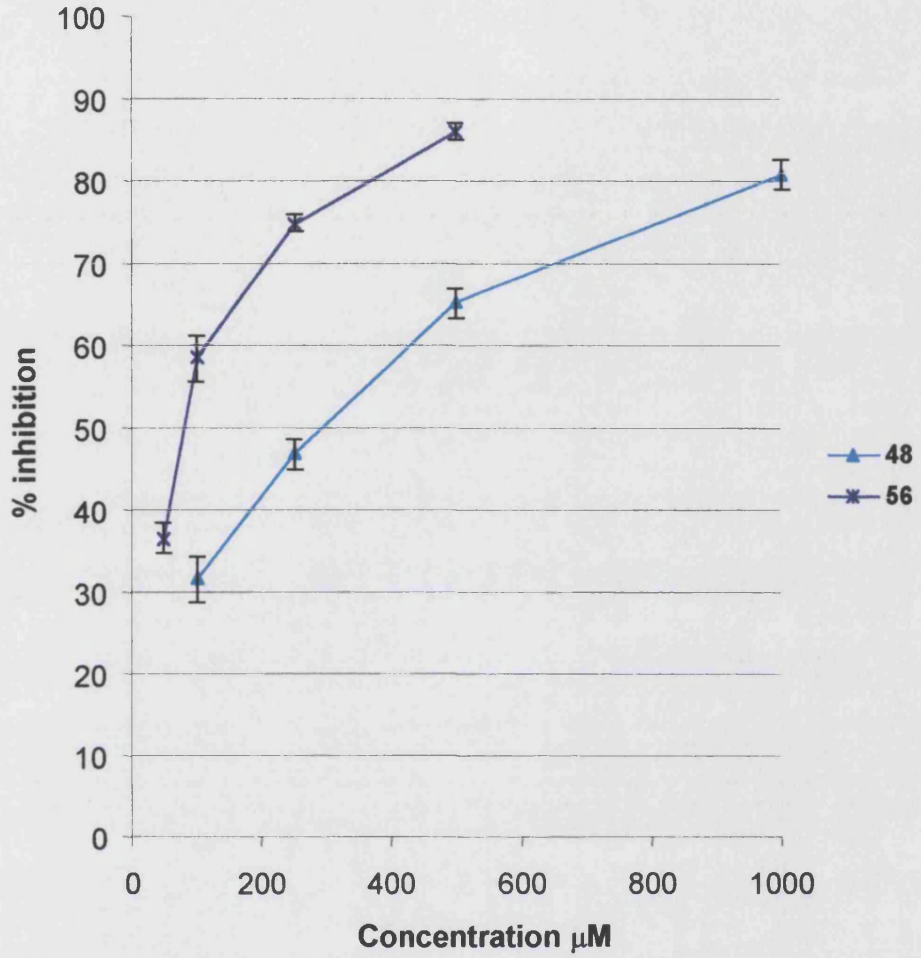
The five most potent compounds were assayed for dose response curves using 10 μM arginine concentration and a fifteen minute pre-incubation time with both isoforms.

The fact that several of these compounds are unaffected by the changes in arginine concentration would suggest that they may be (at least partly) non-competitive inhibitors. It is obvious that more kinetic studies of these target compounds are needed to fully determine the nature and mechanisms of binding.

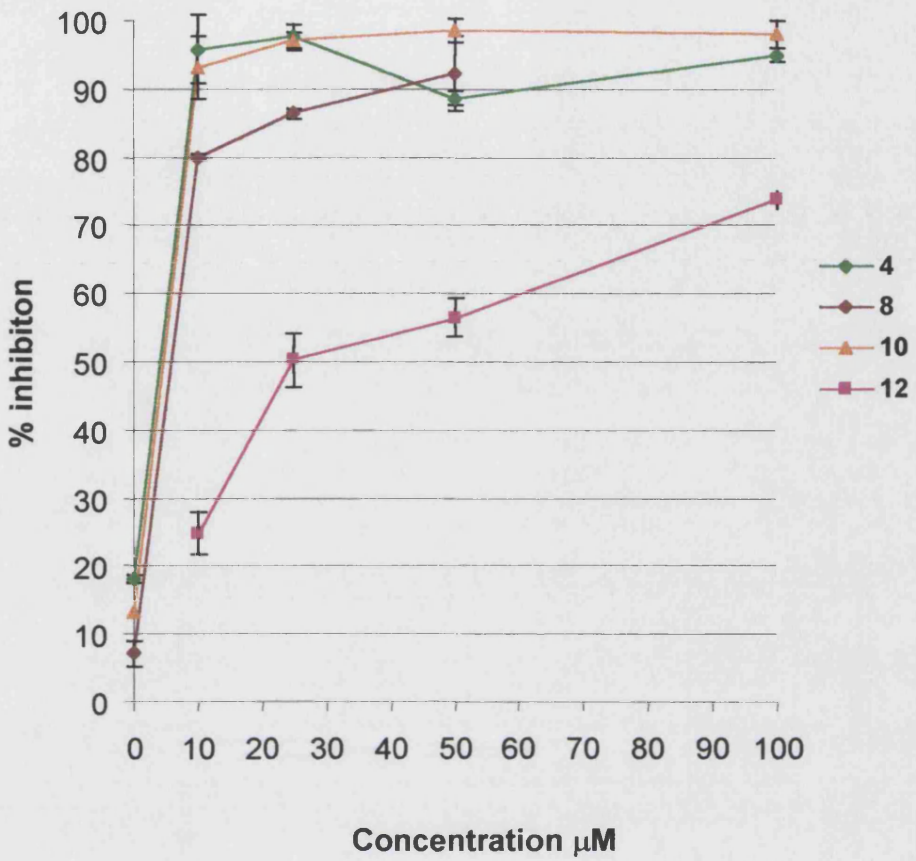
Determination of IC_{50} values for compounds 48, 50, 56, 128 and 129



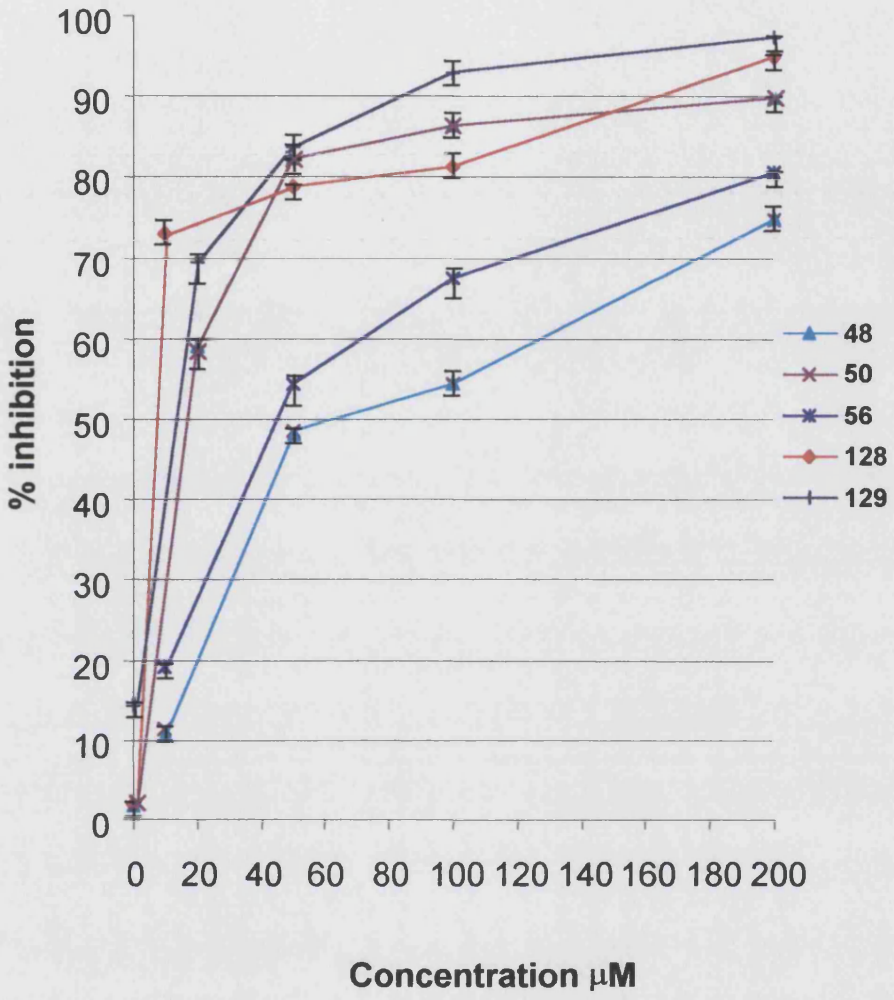
Graph 5.3: Dose response curve for inhibition of hiNOS by compounds **50**, **128** and **129**. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation time of the compound with the enzyme.



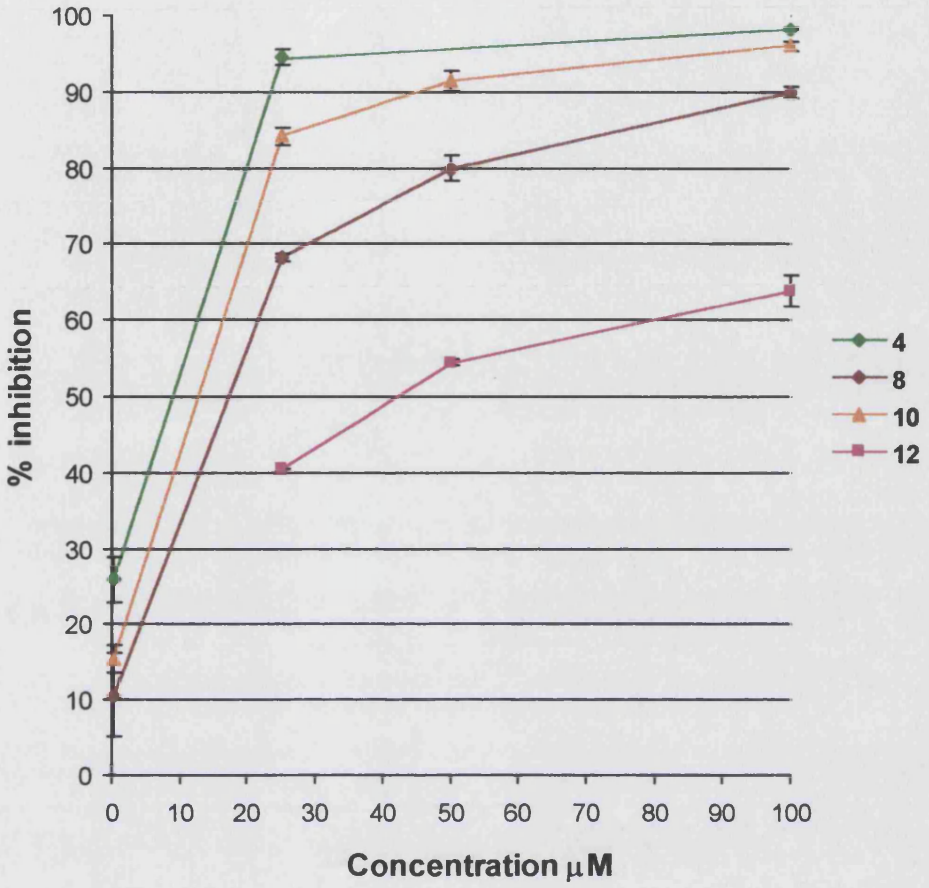
Graph 5.4: Dose response curve for inhibition of hiNOS by compounds **48** and **56**. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation of the compound with the enzyme.



Graph 5.5: Dose response curve for inhibition of hiNOS by known inhibitors **4**, **8**, **10** and **12**. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation of the compound with the enzyme.



Graph 5.6: Dose response curve for inhibition of rat brain cNOS by compounds **48, 50, 56, 128** and **129**. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation of the compound with the enzyme.



Graph 5.7: Dose response curve for inhibition of rat brain cNOS by known inhibitors **4**, **8**, **10** and **12**. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation of the compound with the enzyme.

Compound Number	IC_{50} (μM)		
	hiNOS	cNOS	Selectivity (cNOS/hiNOS)
50	19 ± 2	13 ± 2	
48	260 ± 2	54 ± 1	5 (cNOS)
56	89 ± 2	41 ±2	2 (cNOS)
128	13 ± 1	3 ± 0.5	4 (cNOS)
129	18 ± 0.6	8 ± 0.4	2 (cNOS)
4	< 4 ± 0.5	9 ± 0.5	
12	24 ± 2	40 ± 0.8	
8	< 5 ± 0.5	17 ± 2	
10	< 4 ± 0.5	12 ± 1	

Table 5.11: Comparison of IC_{50} values of compounds **48, 50, 56, 128, 129** and known inhibitors; **4, 12, 8** and **10** against hiNOS and rat brain cNOS. Values are expressed as mean ± SEM (n=3). Assay was carried out with a fifteen minute pre-incubation of the compound with the enzyme.

IC_{50} values for the known inhibitors **4**, **10** and **8** can only be given as < 4, 5 and 4 respectively as insufficient points on the dose response curve for hiNOS were determined close to the IC_{50}.

The two lysine derivatives were the most potent NOS inhibitors. Both compounds showed better IC_{50} results than the known inhibitors **4** and **8** for cNOS. Ideally lead compounds should display at least 100-fold isoform selectivity and possess submicromolar IC_{50} values. As table 5.11 shows, none of the target compounds display selectivity within that range. However, selectivity values given do enable ideas to be considered about how inhibitor structure relates to selectivity and potency. The addition of the methyl ester into the lysine derivative increases the potency and selectivity of the inhibitor for this series. Compound **128** showed a four-fold selectivity for cNOS over iNOS compared to a two-fold selectivity

displayed by **129**. The methyl ester thiourea (**128**) was more potent than the target without the ester functionality (**129**). These results are the reverse of what is seen with compound **7** and its methyl ester **3**. Compound **128** shows an increase in potency for both isoforms when compared to **129**. The methyl ester seems to be an important feature in the design of potent amino acid analogue NOS inhibitors.

The dihydrothiazole target **50** was the most potent of the aromatic targets but showed no isoform selectivity (iNOS 19 μM, cNOS 13 μM). This target is a better non-selective NOS inhibitor than the known inhibitor 7NI. Target **17** (IC_{50} 8.1 and 1.3 μM for iNOS and cNOS respectively (Ulhaq *et al* 1999)) was a lead compound from which target compounds with the dihydrothiazole head group were based. Unfortunately, taking the dihydrothiazole head group and incorporating features of **10** (such as the benzene ring) have not increased the potency and selectivity of the inhibitor.

The benzyl and phenyl thiourea head derivatives gave rise to the most potent NOS inhibitors when compared with the corresponding dihydrothiazoles. Targets **48** and **56** in which the thiourea head group was attached to the benzene ring showed a decline in potency when compared to the lysine thiourea derivatives. Both targets **48** and **56** with the thiourea head group displayed selectivity towards cNOS with the CH_2NH_2 group in the meta position giving rise to the most selective (five-fold compared to two-fold displayed by **56**).

The CH_2NH_2 group gives rise to potent and selective inhibitors. The position and type of the functional group is very important in the design of potent and selective inhibitors. It appears that the dihydrothiazole head group gives rise more potent but non-selective inhibitors while the thiourea head group gives rise to more selective inhibitors. The bridge length between the head group and the remote amine does seem to be important in the design of potent NOS inhibitors. Those targets with seven-atom bridges (**128** and **129**) have given rise to the most potent inhibitors, whereas **50**, **48** and **56** are less potent but display a higher degree of selectivity for cNOS. These findings are consistent with those for **15** which possesses a six-atom bridge and is selective for the nNOS isoform. Unfortunately, the corresponding thiourea with the seven-atom bridge (**35**) showed no potency or selectivity for iNOS

as predicted. This indicates that the acetamidine head group in **10** plays an important role in the selectivity of the inhibitor.

The targets synthesised and tested have given rise to non-selective NOS inhibitors with some compounds **(48, 56, 128)** showing a degree of selectivity for the cNOS isoform. As mentioned in chapter one, the side-effects of cNOS inhibitors outweigh their benefits and so this class of inhibitor has very little use in therapeutics. However, such inhibitors are useful in gaining knowledge of structure-activity relationships and information about binding which enables the design of more selective inhibitors.

Overall structure activity relationships

All three NOS isoforms function as homodimers which comprise two oxygenase domains, linked through interactions between haem domains, and one reductase domain (Siddhanta *et al* 1996). The zinc tetrathiolate centre at the dimer interface helps to stabilise the dimer and form the tetrahydrobiopterin-binding site.

The aromatic targets were based on the known iNOS selective inhibitor **10**. This compound is known to bind to the haem active site through hydrogen-bond interactions with the amidine head group and amino acids Glu363 and Trp358. The benzene ring is positioned above the two propanoate groups of the haem. This is illustrated in figure 5.1.

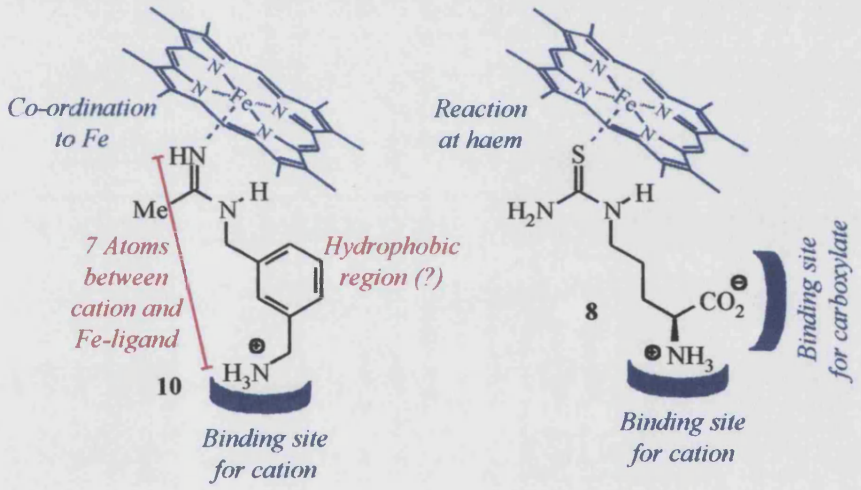


Figure 5.1: Proposed binding of the NOS inhibitors **10** and **8**, illustrating the co-ordination of the amidine and thiourea head groups respectively to the haem.

Developing SAR pictures are particularly beneficial in understanding how target inhibitors may interact with the active site. Also information can be gained about which functional groups give rise to the strongest interactions with the enzyme.

Crystal structures of several different sources of iNOS and eNOS isoforms are available on database and so this information was used to obtain pictures of target inhibitors **48**, **56, 128** and **129** with the human eNOS isoform haem domain.

Due to their similarity in structure, compounds **48** and **56** are believed to inhibit NOS by binding to the L-arginine binding site in a similar conformation to **10**. Figures 5.2 and 5.3 clearly show the sulphur (yellow atom) of the thiourea head group on inhibitors **48** and **56** in close proximity to the haem domain. Target compounds **48** and **56** lack the extra methylene unit between the aromatic ring and the head group functionality when compared with **10**. As a consequence this could place the benzene ring closer to the two propanoate groups of the haem and may be one explanation why **48** and **56** give rise to lower IC_{50} values for cNOS than **10** (ref table 1.5and 5.11).

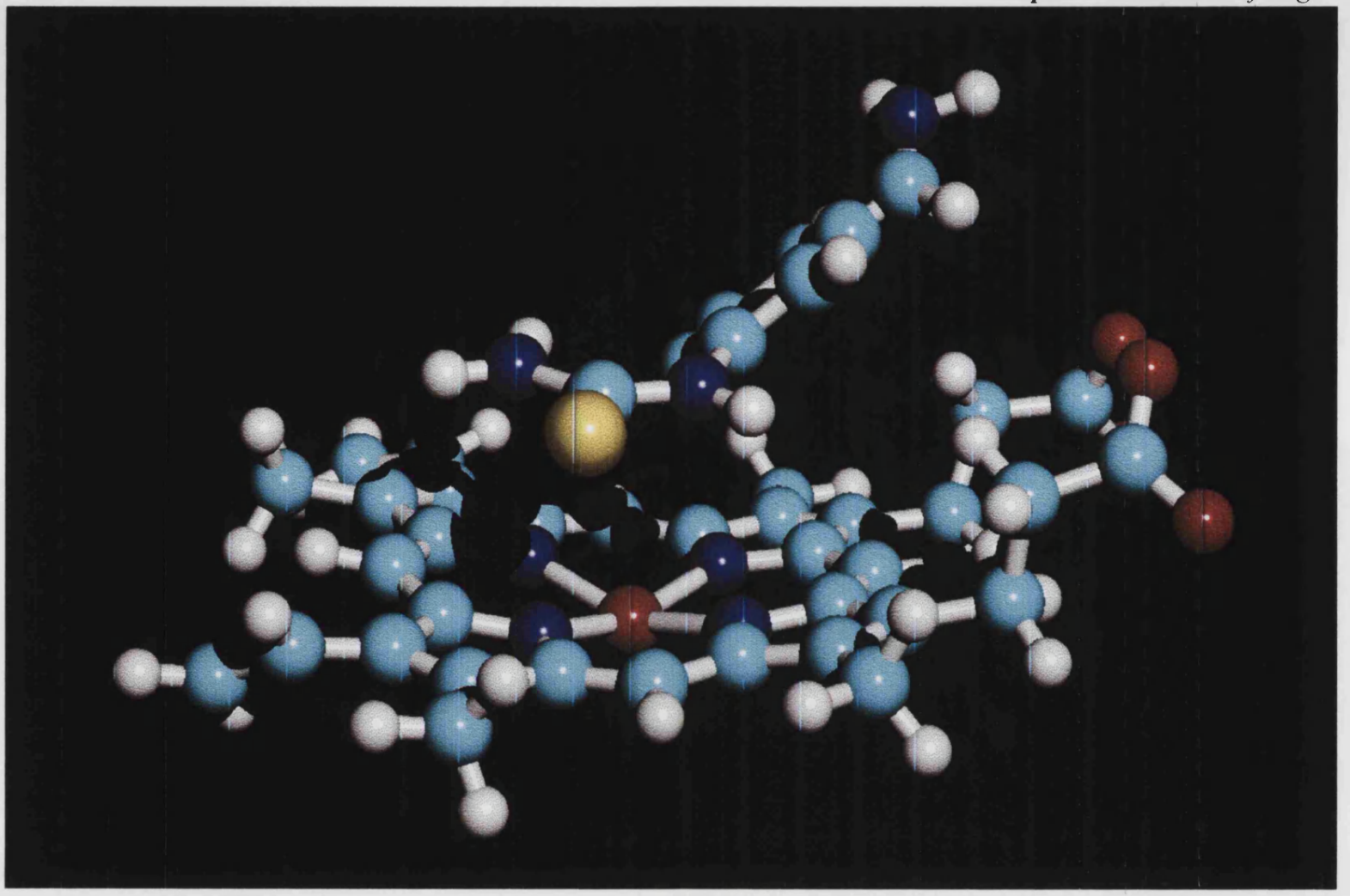


Figure 5.2: Proposed binding of compound **48** to the haem domain of eNOS isoform.

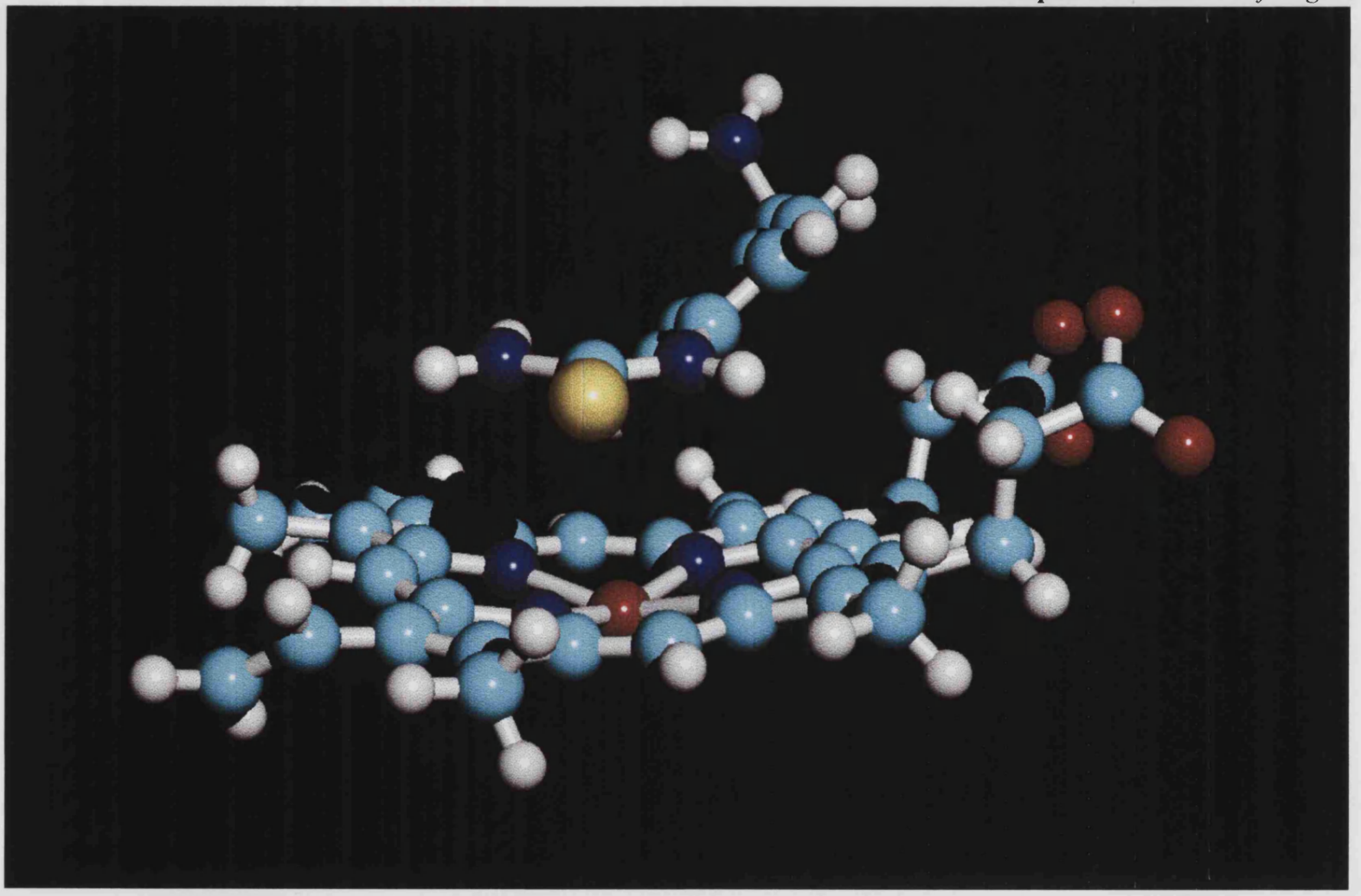


Figure 5.3: Proposed binding of compound **56** to the haem domain of eNOS isoform.

Thiocitrulline binds to the haem domain by interacting with Glu371. The inhibitor is held in place by hydrogen bonds formed between the carboxyl oxygen atom of Glu371 and the sulphur atom in the thiourea group of **8** (Ware and King 2000) (figure 5.1). The sulphur atom is positioned above the haem iron. The lysine targets **128** and **129** are structurally similar to thiocitrulline and so are thought to bind in a like manner. Figures 5.4 and 5.5 show how targets **128** and **129** may interact with the haem domain.

Figure 5.4 and 5.5 show that the amino acid moiety in the lysine targets are much closer to the propanoate groups in the haem active site than targets **48**and **56** which may explain why they are more potent inhibitors. Clearly the head group functionality plays an important role in the potency of the inhibitor by forming interactions with the haem domain. Sulphur is a soft nucleophile and a good ligand for Fe, this may be one explanation why the thiourea targets have given rise to the most potent inhibitors in this series of compounds tested.

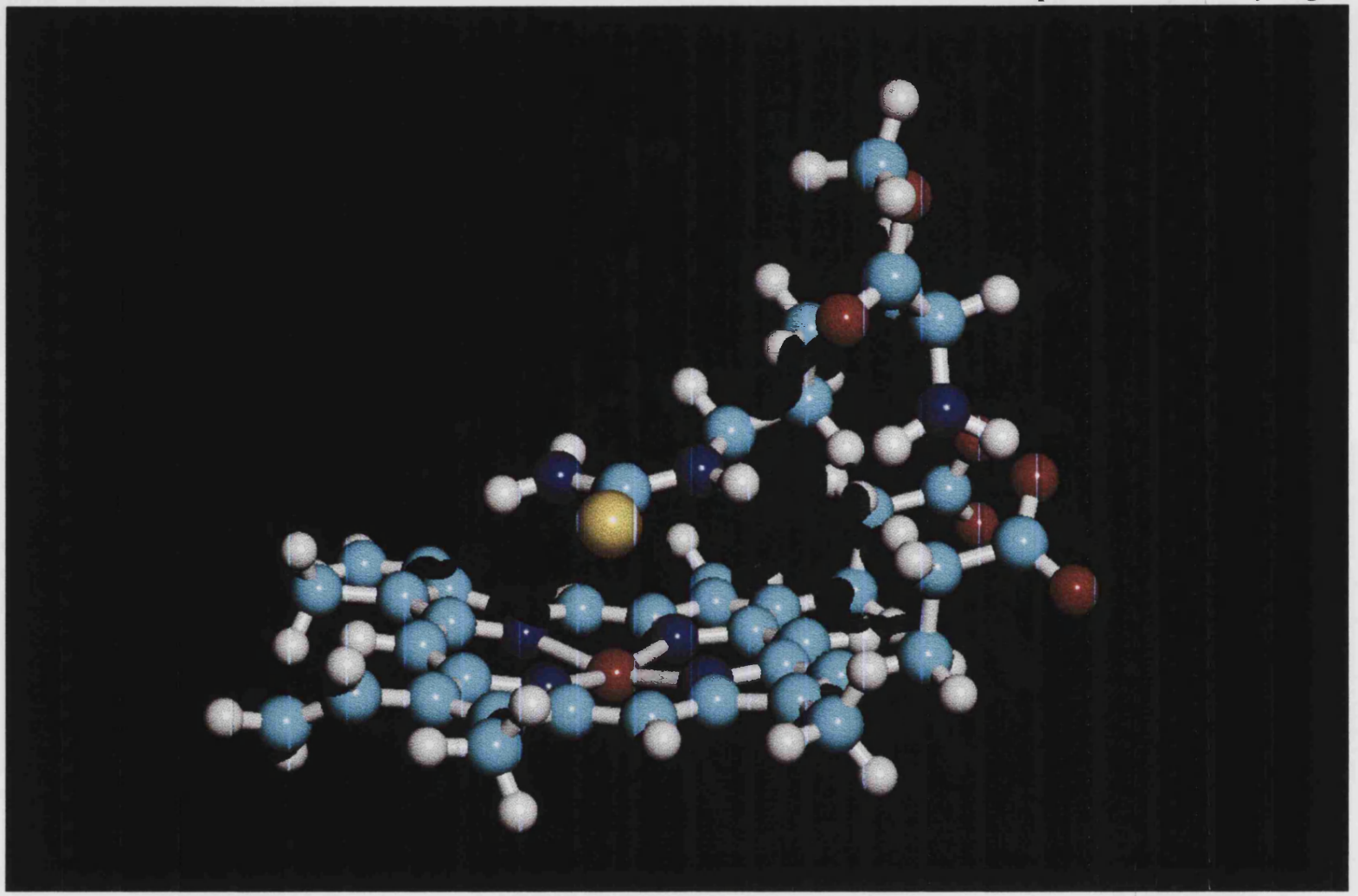


Figure 5.4: Proposed binding of compound **128** to the haem domain of eNOS isoform.

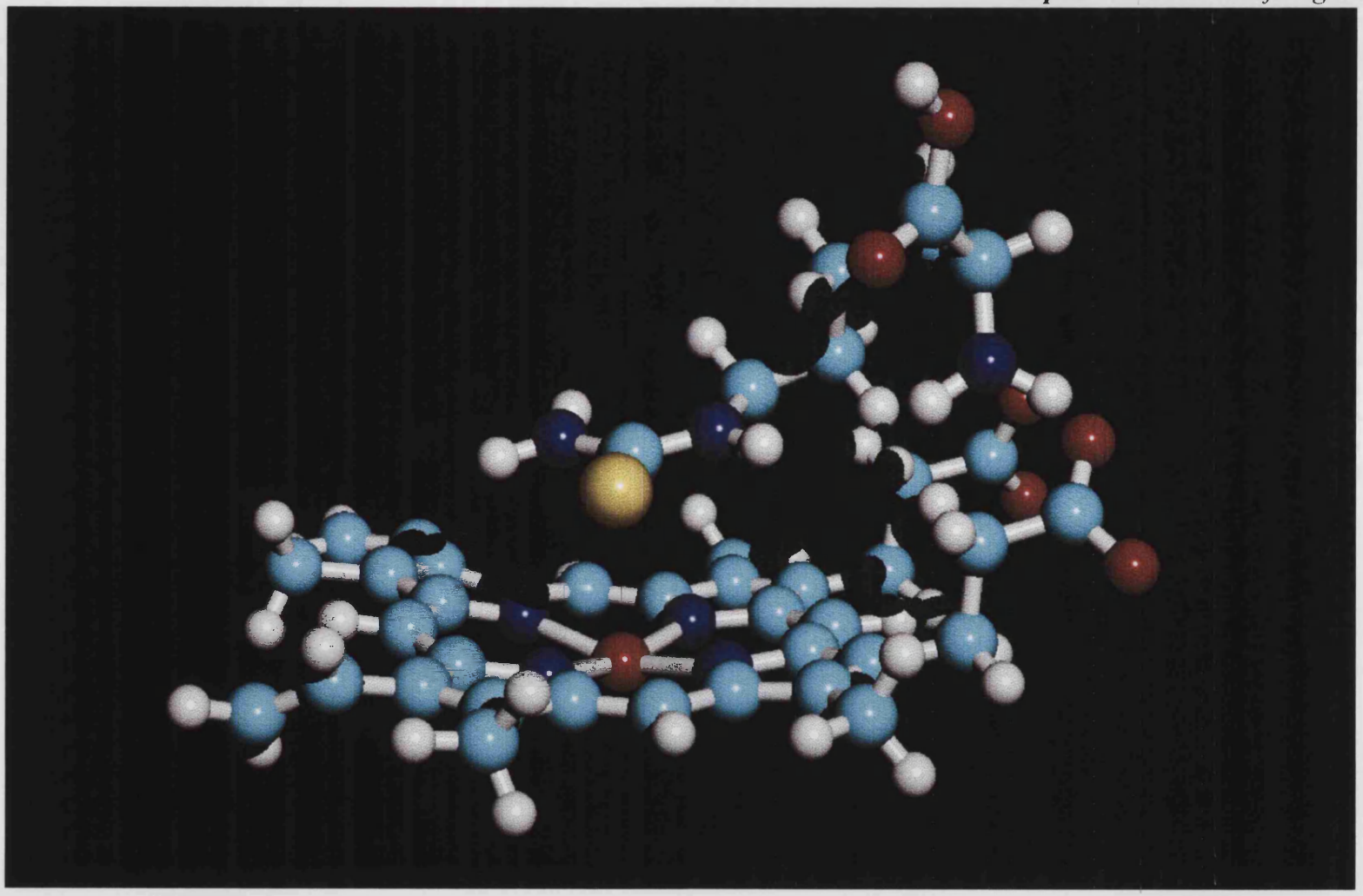


Figure 5.5: Proposed binding of compound **129** to the haem domain of eNOS isoform.

Conclusions

Target compounds **111, 89, 68, 75, 99, 107, 80, 96** and **104** showed the greatest stimulatory values. When the 2-hydroxyethyl motif was introduced into the methoxyarylthiourea targets an increase in the stimulatory values was seen with the iNOS isoform at a pre-incubation time of zero. It appears that this class of compounds can bind at more than one site under different conditions, one site being a stimulatory site (possibly a remote allosteric site) and the other an inhibitory site. Binding to the allosteric site would be stimulatory and fast (which gives rise to stimulation of NO synthesis without pre-incubation), whereas binding to substrate-binding site is slow and inhibitory. The 2-hydroxyethyl intermediates of the aromatic targets possessing the amine and carboxylate functionality would be a good series of compounds to test for NO stimulation. These compounds may have therapeutic use in the development of NO donors, however, the fact that they switch to weak inhibitors with time reduces their clinical potential.

The dihydrothiazole head group gave rise to the most potent NOS inhibitors. Compound **50** was more potent than the known NOS inhibitor **12** but showed no isoform selectivity (19 and 13 μM for iNOS and cNOS, respectively). Aromatic targets with the thiourea head group attached showed the most selectivity for the cNOS isoform. Compound **48** showed five-fold selectivity for the cNOS isoform and gave an IC_{50} value of 54 μM. The most potent and selective aromatic targets possessed the CH_2NH_2 functionality, with the most selective being substituted in the meta position. This functionality provides an optimum size for binding to the NOS active site.

The two lysine derivatives **128** and **129** were the most potent of all the targets tested with **128** displaying a four-fold selectivity for cNOS. Both targets were more potent than **8** and **5** (ref. table 1.2) when tested against the cNOS isoform and as good as **7** when comparing the iNOS isoform (ref. table 1.2). The introduction of the methyl ester to the lysine thiourea target increased the potency of the inhibitor whereas, **3** showed a reduction in potency when compared with **7**.

The therapeutic use of cNOS isoform-selective inhibitors is limited due to the systemic side-effects that could occur. However, such inhibitors are useful in determining SAR.

As seen with all the targets tested, time plays an important role in determining the optimum potency of the inhibitor and also gives an insight into whether the inhibitors display time-dependent kinetics. To develop this project further, it would be useful to determine the nature of inhibition of the most potent and selective inhibitors and ascertain whether they are for example irreversible or reversible with arginine and either competitive or non-competitive.

Experimental

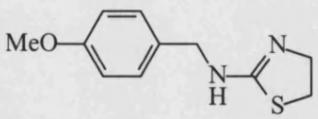
General Synthetic Procedures

^{1}H NMR data were recorded on either JEOL GX 270 or EX 400 spectrometers. Tetramethylsilane was used as an internal standard for ^{1}H NMR samples dissolved in $CDCl_3$ and $(CD_3)_2SO$. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). IR was recorded on a Perkin-Elmer RXI FT-IR spectrometer either a liquid (film), Nujol mull (Nujol) or KBr disc (disc).

FAB Mass Spectra [matrix:-nitrobenzyl alcohol (mNBA)] were recorded at the University of Bath Mass Spectrometry service using a VG Analytical Mass Spectrometer. Microanalysis was carried out at the University of Bath microanalysis service. Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC aluminium silica sheets 60 F_{254}, Art no. 5554). Visualisation was carried out by dipping in ninhydrin (followed by heating) or by u.v. light were appropriate. Solutions in organic solvents were dried with $MgSO_4$ filtered evaporated under reduced pressure. Experiments were conducted at ambient temperature, unless otherwise stated. The aqueous $NaHCO_3$ and the brine were saturated. The stationary phase for the column chromatography was silica gel 33-70 μm.

Melting points were determined using a Reichert-Jung Thermo Galen Kofler block. Chemicals were purchased from the Aldrich Chemical companies unless otherwise stated.

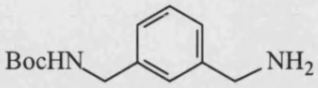
2-(4-Methoxyphenylmethylamino)-4,5-dihydrothiazole 21



4-Methoxybenzylamine **19** (330 mg, 2.4 mmol) was heated with 2-methylthio-4,5-dihydrothiazole **20** (320 mg, 2.4 mmol) at 180°C for 4 h. Evaporation and chromatography (CH_2Cl_2/MeOH 10:1) gave **21** (163 mg, 30%) as white crystals: mp 77-79°C (lit. [Hirashima *et al* 1992] mp 84-86°C); ^{1}H NMR ($CDCl_3$) δ 3.38 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 3.80 (3 H, s, CH_3), 4.04 (2 H, t, J = 7.4 Hz, thiazole 4-H_2),

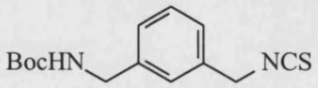
4.40 (2 H, s, CH_2Ar), 6.87 (2 H, d, J = 8.8 Hz, Ar 3,5-H_2), 7.25 (2 H, d, J = 8.8 Hz, Ar 2,6-H_2); IR ν_{max} (disc) 1610 (C=N), 2780 (OCH_3), 3207 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 223.0912 (M + H) ($C_{11}H_{15}N_2OS$ requires 223.0905); Found C, 58.25: H, 6.44; N, 12.35: $C_{11}H_{14}N_2OS$. $0.25H_2O$ requires C, 58.29; H, 6.40; N, 12.36%.

1,1-Dimethylethyl N-(3-(aminomethyl)phenylmethyl)carbamate 23



Di-*tert*-butyl dicarbonate (1.0 g, 4.9 mmol) was added slowly to 1,3-bis(amino-methyl)benzene **22** (2.0 g, 15 mmol) and Et_3N (2.9 g, 29 mmol) in CH_2Cl_2 (15 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation gave **23** (900 mg, 78%) as white crystals: mp 61-64°C (lit. [Callahan *et al* 1989] oil); Rf 0.3 (CH_2Cl_2 /MeOH 3:1); ^1H NMR ($CDCl_3$) δ 1.51 (9 H, s, Bu^t), 1.67 (2 H, s, NH_2), 3.90 (2 H, d, J = 4.3 Hz, CH_2NHBoc), 4.34 (2 H, s, CH_2NH_2), 5.10 (1 H, br, NH), 7.23-7.34 (4 H, m, Ar-H_4); MS (FAB +ve ion) *m/z* 237 (M + H), 181 (M – $Me_2C{=}CH_2$), 164 (M – Bu^tO).

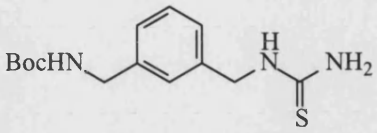
1,1-Dimethylethyl N-(3-(isothiocyanatomethyl)phenylmethyl)carbamate 27



Compound **23** (900 mg, 3.9 mmol), $CaCO_3$ (400 mg, 4.0 mmol), water (3 ml), thiophosgene (900 mg, 7.8 mmol) and $CHCl_3$ (25 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:3) gave **27** (400 mg, 37%) as an oil (lit. [Smith *et al* 1996] mp 43°C); Rf 0.2 (EtOAc/hexane 1:3); ^1H NMR ($CDCl_3$) δ 1.46 (9 H, s, Bu^t), 4.33 (2 H, d, J = 5.6 Hz, CH_2NHBoc), 4.70 (2 H, s, CH_2NCS), 4.91 (1 H, br, NH), 7.22-7.27 (3 H, m, Ar 2,4,6-H_3), 7.34 (1 H, t, J = 7.8 Hz, Ar 5-H); IR ν_{max} (film) 1670 (C=O), 2060 (N=C=S), 3353 (NH) cm^{-1}; MS (FAB +ve ion) *m/z*

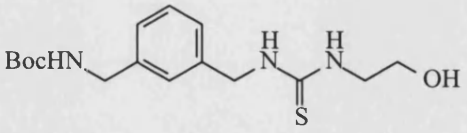
279.1163 (M + H) ($C_{14}H_{19}N_2O_2S$ requires 279.1167), 223 (M – $Me_2C{=}CH_2$), 179 (M – Boc), 164 (M – BocNH).

1,1-Dimethylethyl N-(3-(thioureidomethyl)phenylmethyl)carbamate 28



Ammonia was passed through **27** (400 mg, 1.5 mmol) in CH_2Cl_2 (40 ml) for 30 min. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 4:1) gave **28** (300 mg, 72%) as white crystals: mp 70-72°C; Rf 0.7 (EtOAc/hexane 4:1); ^{1}H NMR ($CDCl_3$) δ 1.40 (9 H, s, Bu^t), 4.18 (2 H, br, CH_2), 4.62 (2 H, br, CH_2), 5.30 (1 H, br, N*H*CSNH_2), 6.01 (2 H, br, NH_2), 7.16-7.29 (5 H, m, Ar-H_4 + NH); IR ν_{max} (disc) 1164 (C=S), 1608 (C=O), 3308 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 296.1426 (M + H) ($C_{14}H_{22}N_3O_2S$ requires 296.1433), 240 (M – $Me_2C{=}CH_2$); Found C, 56.50: H, 7.11: $C_{14}H_{22}N_3O_2S$ requires C, 56.50; H, 7.09%.

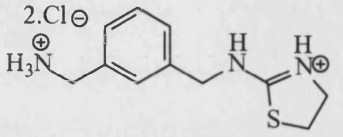
1,1-Dimethylethyl N-(3-(N'-(2-hydroxyethyl)thioureidomethyl)phenylmethyl)-carbamate 30



Compound **27** (210 mg, 1.0 mmol) in acetone (1 ml) was added dropwise during 30 min to 2-aminoethanol (60 mg, 1.0 mmol) in acetone (1 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc/hexane 1:1) gave **30** (90 mg, 30%) as an oil; Rf 0.2 (EtOAc/hexane 1:1); ^{1}H NMR ($(CD_3)_2SO$) δ 1.45 (9 H, s, Bu^t), 1.63 (1 H, br, NH), 3.59 (1 H, br, NH), 3.75 (2 H, m, CH_2NH), 4.01 (2 H, t, *J* = 6.2 Hz, CH_2OH), 4.26 (2 H, m, CH_2Ar), 4.85 (2 H, d, *J* = 4.7 Hz, ArCH_2NBoc), 5.40 (1 H, br, OH) 7.16-7.33 (5 H, m, Ar-H_4 + NH); IR ν_{max} (film) 1170 (C=S), 1642 (C=O), 3413 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 340.1697 (M +

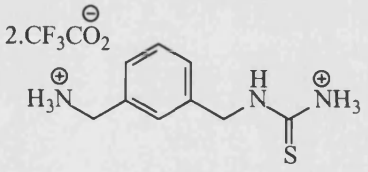
H) ($C_{16}H_{26}N_3O_3S$ requires 340.1695), 284 (M – $Me_2C{=}CH_2$), 179 ($C_9H_{10}N_2S$), 164 (C_9H_9NS).

2-(3-(Aminomethyl)phenylmethylamino)-4,5-dihydrothiazole dihydrochloride 31



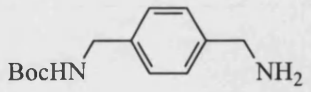
Compound **30** (83 mg, 0.24 mmol) in aq. HCl (6 M, 4 ml) was boiled under reflux for 36 h. Evaporation gave **31** (59 mg, 47%) as an hygroscopic gum; ^{1}H NMR ($CDCl_3$) δ 3.63 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 3.99 (2 H, t, J = 7.4 Hz, thiazole 4-H_2), 4.06 (2 H, brq, J = 5.9 Hz, $CH_2N^+H_3$), 4.77 (2 H, d, J = 6.2 Hz, CH_2Ar), 7.47-7.58 (4 H, m, Ar-H_4), 8.70 (3 H, br, N^+H_3), 10.89 (2 H, s, 2 × NH); IR ν_{max} (film) 1632 (C=N), 3429 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 222.1067 (M + H) ($C_{11}H_{16}N_3S$ requires 222.1065).

N-(3-(Aminomethyl)phenylmethyl)thiourea bis(trifluoroacetate) salt 35



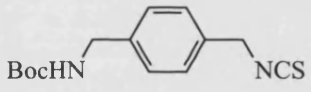
Compound **28** (100 mg, 0.3 mmol) was stirred in trifluoroacetic acid (3 ml) for 5 min. Evaporation gave **35** (140 mg, quant.) as an hygroscopic gum; Rf 0.2 (EtOAc/hexane 4:1); ^{1}H NMR ($(CD_3)_2SO$) δ 4.00 (2 H, m, $CH_2NH_3^+$), 4.65 (2 H, br, CH_2NH), 7.15 (3 H, br, NH_3^+), 7.30-7.34 (4 H, m, Ar-H_4), 8.10 (4 H, br, NH + NH_3^+); IR ν_{max} (film) 1172 (C=S), 1780 (C=O), 3200 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 196.0905 (M + H) ($C_9H_{14}N_3S$ requires 196.0908), 179 (M - NH_3), 162 (M – 2 × NH_3).

1,1-Dimethylethyl N-(4-(aminomethyl)phenylmethyl)carbamate 37



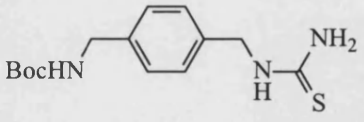
Di-*tert*-butyl dicarbonate (1.0 g, 4.9 mmol) was added slowly to a solution of 1,4-bis-(aminomethyl)benzene **36** (2.0 g, 15 mmol) and Et_3N (2.9 g, 29 mmol) in CH_2Cl_2 (35 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation gave **37** (930 mg, 80%) as an oil: (lit. [Smith *et al* 1996] oil); Rf 0.7 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.46 (9 H, s, Bu^t), 1.52 (2 H, br, NH_2), 3.85 (2 H, s, CH_2NH_2), 4.29 (2 H, d, J = 6.0 Hz, CH_2NHBoc), 5.89 (1 H, br, NH), 7.24-7.28 (4 H, m, Ar-H_4); MS (FAB +ve ion) *m/z* 237 (M + H), 181 (M – $Me_2C{=}CH_2$), 164 (M – Bu^tO).

1,1-Dimethylethyl N-(4-isothiocyanatomethyl)phenylmethyl)carbamate 38



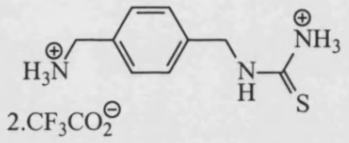
Compound **37** (900 mg, 3.8 mmol), $CaCO_3$ (0.36 g, 3.6 mmol), water (4 ml), thiophosgene (1.1 g, 9.2 mmol) and $CHCl_3$ (30 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:3) gave **38** (860 mg, 81%) as yellow crystals: mp 80-82°C (lit. [Smith *et al* 1996] mp 74°C); Rf 0.4 (EtOAc/hexane 1:3); ^{1}H NMR ($CDCl_3$) δ 1.46 (9 H, s, Bu^t), 4.33 (2 H, m, CH_2NHBoc), 4.70 (2 H, s, CH_2Ar), 4.88 (1 H, br NH), 7.24-7.35 (4 H, m, Ar-H_4); IR ν_{max} (disc) 1682 (C=O), 2091 (N=C=S), 3358 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 279 (M + H), 164 (M – Bu^tO).

1,1-Dimethylethyl N-(4-(thioureidomethyl)phenylmethyl)carbamate 39



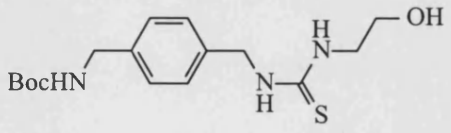
Ammonia was passed through **38** (300 mg, 1.1 mmol) in CH_2Cl_2 (50 ml) for 2 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc) gave **39** (250 mg, 71%) as pale yellow crystals: mp 104-106°C; Rf 0.4 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 1.38 (9 H, s, Bu^t), 4.08 (2 H, d, J = 6.0 Hz, CH_2NHBoc), 4.57 (2 H, br, CH_2Ar), 7.20 (6 H, m, Ar-H_4 + NH_2), 7.37 (1 H, br, NH), 7.98 (1 H, br, NH); IR ν_{max} (disc) 1171 (C=S), 1686 (C=O), 3355 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 296.1423 (M + H) ($C_{14}H_{21}N_3O_3S$ requires 296.1421), 591 (2 M + H), 249 (M – $Me_2C{=}CH_2$); Found C, 56.6: H, 7.09; N, 13.90: $C_{14}H_{22}N_3O_2S$ requires C, 56.72; H, 7.16; N, 14.23%.

N-(4-(Aminomethyl)phenylmethyl)thiourea bis(trifluoroacetate) salt 40



Compound **39** (130 mg, 0.4 mmol) was stirred in trifluoroacetic acid (5 ml) and was stirred for 2 h. Evaporation gave **40** (240 mg, quant.) as white crystals: mp 197-199°C; Rf 0.2 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.99 (4 H, s, 2 × CH_2), 4.64 (3 H, br, NH_3^+), 7.29 (1 H, br, NH), 7.30 (2 H, d, J = 8.0 Hz, Ar 3,5-H_2), 7.37 (2 H, d, J = 8.0 Hz, Ar 2,6-H_2), 8.16 (3 H, br, NH_3^+); IR ν_{max} (disc) 1188 (C=S), 3293 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 196.0920 (M + H) ($C_9H_{14}N_3S$ requires 196.0908), 349 (M + mNBA), 179 (M - NH_3).

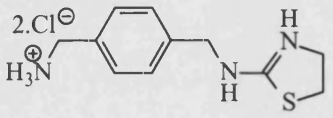
1,1-Dimethylethyl N-(4-(N-(2-hydroxyethyl)thioureidomethyl)phenylmethyl)-carbamate 41



Compound **37** (300 mg, 1.0 mmol) in acetone (1.3 ml) was added dropwise during 30 min to 2-aminoethanol (61 mg, 1.0 mmol) in acetone (1.3 ml). The mixture was boiled under reflux for 4 h. Evaporation gave **41** (140 mg, 41%) as an oil; Rf 0.3

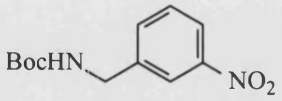
(EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.44 (9 H, s, Bu^t), 3.57 (2 H, br, CH_2NH), 3.68 (2 H, t, J = 4.7 Hz, CH_2OH), 4.24 (2 H, d, J = 5.4 Hz, CH_2NHBoc), 4.67 (2 H, s, CH_2Ar), 5.04 (1 H, s, OH), 6.88 (1 H, br, NH), 7.18 (1 H, br, NH), 7.20 (2 H, d, J = 7.0 Hz, Ar 3,5-H_2), 7.26 (3 H, m, Ar 2,6-H_2 + NH); IR ν_{max} (film) 1171 (C=S), 1682 (C=O), 3297 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 340.1684 (M + H) ($C_{16}H_{26}N_3O_3S$ requires 340.1695), 679 (2 M +H), 284 (M – $Me_2C{=}CH_2$).

2-(4-(Aminomethyl)phenylmethylamino)-4,5-dihydrothiazole dihydrochloride 42



Compound **41** (100 mg, 0.29 mmol) in aq. HCl (6 M, 6 ml) was boiled under reflux for 36 h. Evaporation and recrystallisation (propan-2-ol) gave **42** (40 mg, 47%) as pale yellow crystals: mp 198-200°C; ^{1}H NMR ($(CD_3)_2SO$) δ 3.64 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 4.04 (2 H, m, thiazole 4-H_2), 4.14 (2 H, m, CH_2Ar), 4.61 (2 H, s, $CH_2NH_3^+$), 7.44 (2 H, m, Ar 3,5-H_2), 7.53 (2 H, m, Ar 2,6-H_2); IR ν_{max} (disc) 1654 (C=N), 3425 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 222.1061 (M + H) ($C_{11}H_{16}N_3S$ requires 222.1065), 443 (2 M +H), 375 (M + mNBA), 205 (M – NH_3).

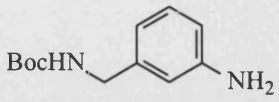
1,1-Dimethylethyl N-(3-nitrophenylmethyl)carbamate 44



Di-*tert*-butyl dicarbonate (1.7 g, 7.8 mmol) was added slowly to 3-nitrobenzylamine **43** (1.0 g, 6.6 mmol) and Et_3N (1.1 g, 11 mmol) in CH_2Cl_2 (25 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated and the residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation and chromatography (CH_2Cl_2) gave **44** (900 mg, 56%) as white crystals: mp 124-126°C (lit. [Collins *et al* 1998] no mp given); Rf 0.3 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.39 (9 H, s, Bu^t), 4.35 (2 H, d, J = 6.1 Hz, CH_2), 5.23 (1 H, br, NH), 7.40 (1 H, dd, J = 8.9, 7.8 Hz, Ar 5-H),

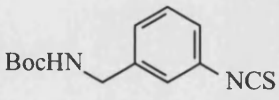
7.54 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.03 (1 H, d, J = 8.9 Hz, Ar 4-H), 8.04 (1 H, s, Ar 2-H); MS (FAB +ve ion) *m/z* 253.1184 (M + H) ($C_{12}H_{17}N_2O_4$ requires 253.1188), 197 (M - $Me_2C{=}CH_2$); Found C, 57.30: H, 6.35; N, 11.20: $C_{12}H_{15}N_2O_4$ requires C, 57.14; H, 6.35; N, 11.11%.

1,1-Dimethylethyl N-(3-aminophenylmethyl)carbamate 45



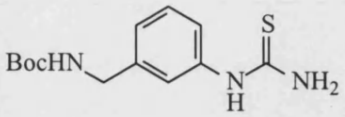
Compound **44** (900 mg, 3.7 mmol) and $SnCl_2.2H_2O$ (2.6 g, 10 mmol) in EtOH (8 ml) were boiled under reflux for 30 min under N_2. The solution was cooled to 0°C and made basic with 5% aq. $NaHCO_3$, before being extracted with EtOAc. The extract was washed with brine and dried. Evaporation gave **45** (250 mg, 31%) as an oil (lit. [Collins *et al* 1998] oil); Rf 0.4 (EtOAc/$CHCl_3$ 1:1); ^{1}H NMR ($CDCl_3$) δ 1.40 (9 H, s, Bu^t), 3.59 (2 H, br, NH_2), 4.15 (2 H, d, J = 5.7 Hz, CH_2), 4.70 (1 H, br, NH), 6.55 (3 H, m, Ar 2,4,6-H_3), 7.06 (1 H t, J = 7.7 Hz, Ar 5-H); MS (FAB +ve) *m/z* 222 (M), 167 (M -$Me_2C{=}CH_2$), 121 (M - Boc), 106 (M - BocNH).

1,1-Dimethylethyl *N*-(3-isothiocyanatophenylmethyl)carbamate 46



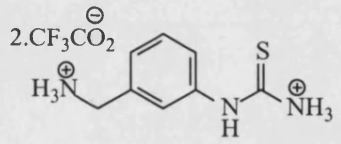
Compound **45** (200 mg, 0.9 mmol), $CaCO_3$ (77 mg, 0.8 mmol), water (1 ml), thiophosgene (200 mg, 1.8 mmol) and $CHCl_3$ (5 ml) were stirred vigorously for 48 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:3) gave **46** (130 mg, 70%) as white crystals: mp 70-72°C; Rf 0.5 (EtOAc/hexane 1:3); ^{1}H NMR ($(CD_3)_2SO$) δ 1.39 (9 H, s, Bu^t), 4.12 (2 H, d, J = 5.9 Hz, CH_2), 7.23 (3 H, m, Ar 2,4,6-H_3), 7.43 (1 H, t, J = 7.7 Hz, Ar 5-H), 7.46 (1 H, br, NH); IR ν_{max} (disc) 1675 (C=O), 2100 (N=C=S), 3180 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 265.1012 (M + H) ($C_{13}H_{17}N_2O_2S$ requires 265.1011), 209 (M – $Me_2C{=}CH_2$); Found C, 58.50: H, 6.02; N, 10.40: $C_{13}H_{16}N_2O_2S$ requires C, 59.00; H, 6.06; N, 10.60%.

1,1-Dimethylethyl N-(3-thioureidophenylmethyl)carbamate 47



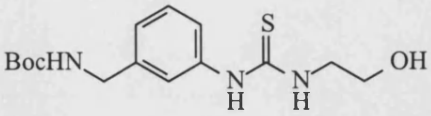
Ammonia was passed through **46** (200 mg, 0.8 mmol) in CH_2Cl_2 (20 ml) for 30 min. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 4:1) gave **47** (85 mg, 38%) as yellow crystals: mp 198-200°C; Rf 0.2 (EtOAc/hexane 4:1); ^{1}H NMR ($CDCl_3$) δ 1.37 (9 H, s, Bu^t), 4.01 (2 H, d, J = 7.4 Hz, CH_2), 6.96 (1 H, t, J = 7.4 Hz, NH), 7.23 (1 H, d, J = 7.8 Hz, Ar-H), 7.28-7.35 (5 H, m, Ar-H_3 + NH_2), 9.78 (1 H, s, NH); IR ν_{max} (disc) 1173 (C=S), 3291 (NH) cm^{-1}, MS (FAB +ve ion) *m/z* 282.1295 (M + H) ($C_{13}H_{20}N_3O_2S$ requires 282.1276), 226 (M – $Me_2C{=}CH_2$).

N-(3-Aminomethylphenyl)thiourea bis(trifluoroacetate) salt 48



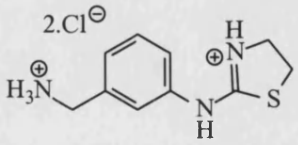
Compound **47** (85 mg, 0.3 mmol) was taken up in trifluoroacetic acid (3 ml) and was stirred for 5 min. Evaporation gave **48** (75 mg, quant.) as an hygroscopic gum; ^{1}H NMR ($(CD_3)_2SO$) δ 4.02 (2 H, brq, J = 5.9 Hz, CH_2), 7.09 (3 H, br, NH_3^+), 7.20 (1 H, m, Ar 4-H), 7.40 (2 H, m, Ar 5,6-H_2), 7.50 (1 H, s, Ar 2-H), 8.16 (3 H, br, N^+H_3), 9.87 (1 H, s, NH); ^{13}C NMR ($(CD_3)_2SO$) δ 42.3 (CH_2), 123.4 (C-2) 123.5 (C-6), 124.8 (C-4), 129.1 (C-5), 134.5 (C-1), 139.5 (C-3), 181.1 (C=S); IR ν_{max} (film) 1170 (C=S), 3407 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 182.0768 (M + H) ($C_8H_{12}N_3S$ requires 182.0752), 164 (M – NH_3^+).

1,1-Dimethylethyl N-(3-(N'-(2-hydroxyethyl)thioureido)phenylmethyl) carbamate 49



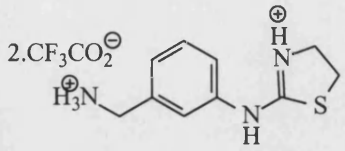
Compound **46** (200 mg, 0.7 mmol) in acetone (1.0 ml) was added dropwise during 30 min to 2-aminoethanol (43 mg, 0.7 mmol) in acetone (1.0 ml). The mixture was boiled under reflux for 2 h. Evaporation and chromatography (EtOAc) gave **49** (103 mg, 43%) as an oil; Rf 0.3 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 1.39 (9 H, s, Bu^t), 3.50 (2 H, br, CH_2NH), 3.52 (2 H, m, CH_2OH), 4.04 (2 H, d, J = 6.6 Hz, CH_2NHBoc), 4.80 (1 H, s, OH), 6.96 (1 H, d, J = 7.4 Hz, Ar 4-H), 7.23 (2 H, m, NH + Ar 2-H), 7.36 (2 H, m, Ar 5,6-H_2), 7.66 (1 H, br, NH), 9.60 (1 H, br, NH); IR ν_{max} (film) 1164 (C=S), 1693 (C=O), 3380 (NH); MS (FAB +ve ion) *m/z* 326.1541 (M + H) ($C_{15}H_{24}N_3O_3S$ requires 326.1538), 651 (2 M + H), 270 (M – $Me_2C{=}CH_2$).

2-(3-(Aminomethyl)phenylamino)-4,5-dihydrothiazole dihydrochloride 50



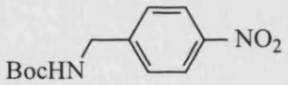
Compound **49** (830 mg, 2.6 mmol) was taken up in aq. HCl (6 M, 3 ml) and was boiled under reflux for 43 h. Evaporation gave **50** (820 mg, quant.) as buff crystals: mp 208-210°C; ^{1}H NMR ($(CD_3)_2SO$) δ 3.65 (2 H, t, J = 7.7 Hz, thiazole 5-H_2), 4.01 (2 H, t, J = 7.7 Hz, thiazole 4-H_2), 4.11 (2 H, m, $CH_2NH_3^+$), 7.39 (1 H, d, J = 7.5 Hz, Ar 4-H), 7.56 (2 H, m, Ar 5,6-H_2), 7.62 (1 H, s, Ar 2-H), 8.66 (3 H, s, N^+H_3), 10.5 (1 H, br, NH), 12.7 (1 H, br, NH); IR ν_{max} (disc) 1640 (C=N), 3432 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0918 (M + H) ($C_{10}H_{14}N_3S$ requires 208.0908); Found C, 37.91: H, 6.06; N, 13.29: $C_{10}H_{13}N_3S$ $2H_2O$ 2HCl requires C, 38.50; H, 5.67; N, 13.00%.

2-(3-Aminomethylphenylamino-4,5-dihydrothiazole bis(trifluoroacetate) salt 50a



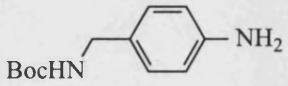
Compound **49** (103 mg, 0.3 mmol) was taken up in trifluoroacetic acid (3 ml) and was stirred for 5 min. Evaporation gave **50a** (138 mg, quant.) as an hygroscopic gum; ^{1}H NMR ($(CD_3)_2SO$) δ 3.62 (2 H, t, J = 7.7 Hz, thiazole 5-H_2), 4.0 (4 H, m, thiazole 4-H_2 + CH_2Ar), 7.30 (4 H, m, Ar-H_4), 7.86 (1 H, br, NH), 8.18 (3 H, m, N^+H_3), 9.73 (1 H, br, NH); IR ν_{max} (film) 1674 (C=N), 3398 (NH); MS (FAB +ve ion) *m/z* 208.0915 (M + H) ($C_{10}H_{14}N_3S$ requires 208.0908), 191 (M - NH_3^+).

1,1-Dimethylethyl N-(4-nitrophenylmethyl)carbamate 52



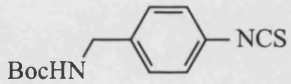
Di-*tert*-butyl dicarbonate (3.3 g, 15 mmol) was added slowly to 4-nitrobenzylamine **51** (2.0 g, 11 mmol) and Et_3N (2.1 g, 21 mmol), in CH_2Cl_2 (25 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation gave **52** (2.4 g, 91%) as white crystals: mp 111-114°C (lit. [Tanaka *et al* 1988] mp 109-110°C); Rf 0.6 (EtOAc/hexane 2:1); ^{1}H NMR ($CDCl_3$) δ 1.53 (9 H, s, Bu^t), 4.42 (2 H, d, J = 5.9 Hz, CH_2), 5.05 (1 H, br, NH), 7.44 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2), 8.19 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2); MS (FAB +ve) *m/z* 505 (2 M + H), 406 (2 M + H – Boc), 275 (M + Na), 253 (M + H), 197 (M – $Me_2C{=}CH_2$), 180 (M – Bu^tO).

1,1-Dimethylethyl *N*-(4-aminophenylmethyl)carbamate 53



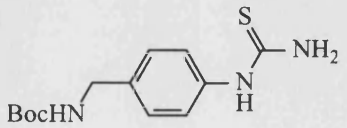
Compound **52** (2.0 g 7.8 mmol) was boiled under reflux with $SnCl_2.2H_2O$ (7.4 g, 39 mmol) in EtOH (17 ml) for 1 h. The solution was cooled to 0°C, made basic with 5% aq. $NaHCO_3$, extracted with EtOAc, washed with brine and dried. Evaporation gave **53** (250 mg, 31%) as an oil: (lit. [Liu *et al* 1998] mp 87-88°C); Rf 0.5 (EtOAc/hexane 2:1); ^{1}H NMR ($CDCl_3$) δ 1.45 (9 H s Bu^t), 3.8 (2 H, br, NH_2), 4.18 (2 H, d, J = 5.1 Hz, CH_2), 4.73 (1 H, br, NH), 6.64 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.07 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2); MS (FAB +ve) *m/z* 222 (M + H), 445 (2 M + H), 165 (M – $Me_2C{=}CH_2$).

1,1-Dimethylethyl N-(4-isothiocyanatophenylmethyl)carbamate 54



Compound **53** (900 mg, 3.9 mmol), $CaCO_3$ (0.4 g, 0.4 mmol) water (3 ml) and thiophosgene (400 mg, 7.8 mmol) and $CHCl_3$ (25 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:3) gave **54** (260 mg, 26%) as a pale yellow powder: mp 113-115°C; Rf 0.4 (EtOAc/hexane 1:3); ^{1}H NMR ($CDCl_3$) δ 1.46 (9 H, s, Bu^t), 4.29 (2 H, d, J = 5.6 Hz, CH_2), 4.91 (1 H, br, NH), 7.18 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2), 7.27 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2); IR ν_{max} (disc) 1683 (C=O), 2123 (N=C=S), 3366 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 265.1006 (M + H) ($C_{13}H_{17}N_2O_2S$ requires 265.1011), 529 (2 M +H), 209 (M – $Me_2C{=}CH_2$); Found C, 58.10: H, 5.92; N, 10.30: $C_{13}H_{16}N_2O_2S$ 0.25 H_2O requires C, 58.08; H, 6.19; N, 10.42%.

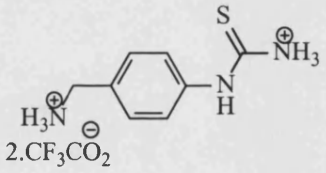
1,1-Dimethylethyl N-(4-thioureidophenylmethyl)carbamate 55



Ammonia was passed through a solution of **54** (400 mg, 1.5 mmol) in CH_2Cl_2 (40 ml) for 30 min. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 4:1) gave **55** (250 mg, 66%) as white crystals: mp

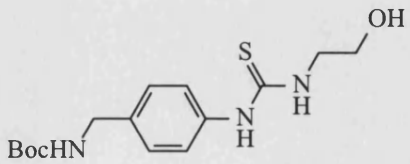
89-91°C; Rf 0.3 (EtOAc/hexane 4:1); ^{1}H NMR ($CDCl_3$) δ 1.46 (9 H, s, Bu^t), 4.30 (2 H, d, J = 6.0 Hz, CH_2), 5.03 (1 H, br, NH), 6.25 (2 H, br, NH_2), 7.19 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.33 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 8.39 (1 H, br, NH); IR ν_{max} (disc) 1187 (C=S), 1690 (C=O), 3298 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 282.1276 (M + H) ($C_{13}H_{20}N_3O_2S$ requires 282.1276), 226 (M – $Me_2C{=}CH_2$); Found C, 54.40: H, 6.73; N, 14.20: $C_{13}H_{19}N_3O_2S$ 0.5 H_2O requires C, 53.87; H, 6.78; N, 14.49%.

N-(4-Aminomethylphenyl)thiourea bis(trifluoroacetate) salt 56



Compound **55** (52 mg, 0.18 mmol) was stirred in trifluoroacetic acid (3 ml) for 2 h. Evaporation gave **56** (880 mg, quant.) as an hygroscopic gum; ^{1}H NMR ($(CD_3)_2SO$) δ 3.96 (2 H, q, J = 5.6 Hz, CH_2), 7.38 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.51 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.51 (3 H, br, NH_3^+), 8.11 (3 H, br, NH_3^+), 9.83 (1 H, s, NH); IR ν_{max} (film) 1173 (C=S), 3369 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 182.0748 (M + H) ($C_8H_{12}N_3S$ requires 182.0752), 335 (M + mNBA), 164 (M – NH_3).

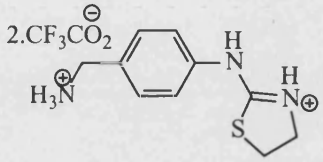
1,1-Dimethylethyl N-(4-N'-(2-hydroxyethyl)thioureido)phenylmethyl)carbamate 57



Compound **54** (120 mg, 0.5 mmol) in acetone (0.6 ml) was added dropwise during 30 min to 2-aminoethanol (28 mg, 0.5 mmol) in acetone (0.6 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **57** (67 mg, 45%) as an oil; Rf 0.5 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 1.39 (9 H, s, Bu^t), 3.32 (2 H, m, CH_2NH), 3.52 (2 H, m, CH_2OH), 4.07 (2 H, d, J = 6.2 Hz, CH_2NHBoc),

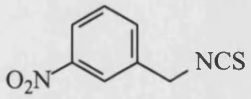
4.94 (1 H, br, OH), 7.15 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.33 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.38 (1 H, br, NH), 7.65 (1 H, br, NH), 9.57 (1 H, br, NH); IR ν_{max} (film) 1166 (C=S), 1689 (C=O), 3323 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 326.1552 (M + H) ($C_{15}H_{24}N_3O_3S$ requires 326.1538).

2-(4-(Aminomethylphenyl)-4,5 dihydrothiazole) bis(trifluoroacetate) salt 58



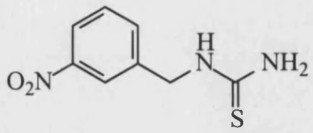
Compound **57** (57 mg 0.18 mmol) was stirred in trifluoroacetic acid (3 ml) for 2 h. Evaporation gave **58** (880 mg quant.) as an hygroscopic gum; ^{1}H NMR ($(CD_3)_2SO$) δ 3.60 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 4.00 (2 H, t, J = 7.4 Hz, thiazole 4-H_2), 4.03 (2 H, q, J = 6.0 Hz, CH_2NH_3), 4.59 (1 H, br, NH), 7.37 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.54 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 8.26 (3 H, br, NH_3^+); IR ν_{max} (film) 1643 (C=N), 3400 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0914 (M + H) ($C_{10}H_{14}N_3S$ requires 208.0908), 191 (M – NH_3).

3-Nitrophenylmethylisothiocyanate 62



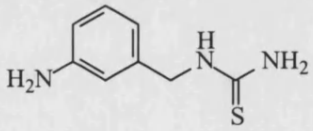
Compound **43** (500 mg, 3.3 mmol), $CaCO_3$ (0.3 g, 3.0 mmol), water (4 ml) thiophosgene (700 mg, 6.6 mmol) and $CHCl_3$ (25 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc) gave **62** (390 mg, 61%) as yellow crystals: mp 65-67°C (lit. [Hirashima *et al* 1992] oil); Rf 0.7 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 4.82 (2 H, s, CH_2), 7.62 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.69 (1 H, d, J = 7.8, Hz, Ar 4-H), 8.20 (1 H, s, Ar 2-H), 8.21 (1 H, d, J = 7.8 Hz, Ar 6-H); IR ν_{max} (disc) 1347 (NO_2), 1526 (NO_2), 2135 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 193 (M – H), 347 (M – H + mNBA).

N-(3-Nitrophenylmethyl)thiourea 63



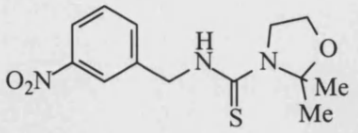
Ammonia was passed through **62** (150 mg, 0.7 mmol) in CH_2Cl_2 (20 ml) for 40 min. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc) gave **63** (110 mg, 68%) as yellow crystals: mp 143-145°C; Rf 0.5 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 4.77 (2 H, s, CH_2), 7.20 (2 H, br, NH_2), 7.64 (1 H, dd, J = 8.2, 7.8 Hz, Ar 5-H), 7.74 (1 H, d, J = 7.8 Hz, Ar 4-H), 8.10 (1 H, s, Ar 2-H), 8.11 (1 H, d, J = 8.2 Hz, Ar 6-H); IR ν_{max} (disc) 1159 (C=S), 1347 (NO_2), 1529 (NO_2), 3292 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 212.0498 (M + H) ($C_8H_{10}N_3O_2S$ requires 212.0494), 365 (M + mNBA), 196 (M – NH_2).

N-(3-Aminophenylmethyl)thiourea 64



Compound **63** (90 mg 0.4 mmol) was boiled under reflux with $SnCl_2.2H_2O$ (0.2 g, 1.2 mmol), in EtOH (5 ml) for 1 h. The solution was cooled to 0°C, made basic with 5% aq. $NaHCO_3$, extracted with EtOAc, washed with brine and dried. Evaporation and chromatography (EtOAc/hexane 5:1) gave **64** (40 mg, 55%) as buff crystals: mp 141-143°C; Rf 0.3 (EtOAc/hexane 5:1); ^{1}H NMR (CD_3OD) δ 3.34 (2 H, s, CH_2), 4.25 (1 H, br, NH), 4.58 (2 H, br, NH_2), 6.62 (3 H, m, Ar 2,4,6-H_3), 6.68 (1 H, br, NH), 7.05 (1 H, t, J = 7.8 Hz, Ar 5-H); IR ν_{max} (disc) 1166 (C=S), 3289 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 182.0757 (M + H) ($C_8H_{12}N_3S$ requires 182.0752), 335 (M + mNBA).

2,2-Dimethyl-3-(N-(3-nitrophenylmethyl)aminothiocarbonyl)tetrahydrooxazole 65 and **N-(2-hydroxyethyl)-N'-(3-nitrophenylmethyl)thiourea 66**



(66) **(65)**

Compound **62** (200 mg, 1.0 mmol) in acetone (1.2 ml) was added dropwise during 30 min to 2-aminoethanol (61 mg, 1.0 mmol) in acetone (1.2 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **65** (50 mg, 19%) as an oil; Rf 0.6 (EtOAc); and **66** (180 mg, 70%) as buff crystals: mp 79-81°C; Rf 0.4 (EtOAc);

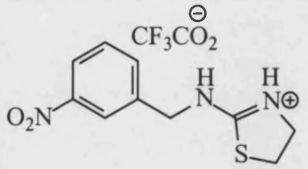
Compound 65

^{1}H NMR ($CDCl_3$) δ 1.80 (6 H, s, 2 × CH_3), 3.82 (2 H, br, oxazole 4-H_2), 4.05 (4 H, t, J = 6.6 Hz, oxazole 5-H_2), 5.02 (2 H, d, J = 5.4 Hz, CH_2Ar), 5.70 (1 H, br, NH), 7.55 (1 H, dd, J = 8.2, 7.8 Hz, Ar 5-H), 7.72 (1 H, d, J = 8.0 Hz, Ar 4-H), 8.15 (1 H, d, J = 8.0 Hz, Ar 6-H), 8.16 (1 H, s, Ar 2-H); IR ν_{max} (film) 1144 (C=S), 1346 (NO_2), 1534 (NO_2), 3413 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 296.1066 (M + H) ($C_{13}H_{18}N_3O_3S$ requires 296.1069), 449 (M + mNBA).

Compound 66

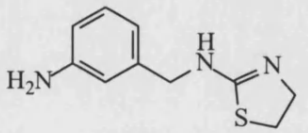
^{1}H NMR ($CDCl_3$) δ 3.61 (2 H, br, CH_2NH), 3.82 (2 H, t, J = 4.7 Hz, CH_2OH), 4.89 (2 H, d, J = 5.4 Hz, CH_2Ar), 6.78 (1 H, br, OH), 7.28 (1 H, br, NH), 7.50 (1 H, t, J = 7.4 Hz, Ar 5-H), 7.77 (1 H, d, J = 7.4 Hz, Ar 4-H), 8.11 (1 H, d, J = 7.4 Hz, Ar 6-H), 8.16 (1 H, s, Ar 2-H); IR ν_{max} (disc) 1144 (C=S), 1348 (NO_2), 1531 (NO_2), 3402 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 256.0753 (M + H) ($C_{10}H_{14}N_3O_3S$ requires 256.0756), 409 (M + mNBA).

2-(3-Nitrophenylmethylamino)-4,5-dihydrothiazole trifluoroacetate salt 67



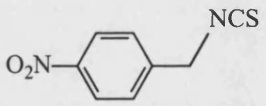
Compound **65** (120 mg, 0.47 mmol) was taken up in trifluoroacetic acid (5 ml) and stirred for 16 h. Evaporation gave **67** (170 mg, quant.) as an oil: (lit. [Hirashima *et al* 1992] mp 91-92°C); Rf 0.6 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.55 (2 H, t, J = 7.8 Hz, thiazole 5-H_2), 4.05 (2 H, t, J = 7.8 Hz, thiazole 4-H_2), 4.59 (2 H, d, J = 4.7 Hz, CH_2Ar), 7.61 (1 H, dd, J = 8.2, 7.8 Hz, Ar 5-H), 7.69 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.17 (1 H, s, Ar 2-H), 8.21 (1 H, d, J = 8.2 Hz, Ar 4-H), 11.94 (1 H, br, NH), 12.32 (1 H, br NH); IR ν_{max} (film) 1352 (NO_2), 1532 (NO_2), 1679 (C=N), 3170 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 238.0640 (M + H) ($C_{10}H_{12}N_3O_2S$ requires 238.0650), 475 (2 M + H).

2-(3-Aminophenylmethylamino)-4,5-dihydrothiazole 68



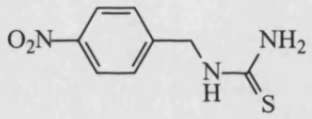
Compound **67** (150 mg, 0.6 mmol) was boiled under reflux with $SnCl_2.2H_2O$ (0.3 g 1.7 mmol), in EtOH (8 ml) for 2 h. The solution was cooled at 0°C, made basic with 5% aq. $NaHCO_3$, extracted with EtOAc, washed with brine and dried. Evaporation gave **68** (150 mg, quant.) as an oil; ^{1}H NMR (CD_3OD) δ 3.30 (2 H, m, thiazole 5-H_2), 3.90 (2 H, m, thiazole 4-H_2), 4.30 (2 H, br, CH_2Ar), 6.62 (2 H, m, Ar 4,6-H_2), 6.67 (1 H, s, Ar 2-H), 7.04 (1 H, t, J = 7.4 Hz, Ar 5-H); IR ν_{max} (film) 1618 (C=N); 3391 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0905 (M + H) ($C_{10}H_{14}N_3S$ requires 208.0908), 196 (C_7H_8N).

4-Nitrophenylmethylisothiocyanate 69



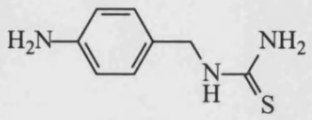
Compound **51** (1.0 g, 6.5 mmol), $CaCO_3$ (500 mg, 5.0 mmol), water (4 ml) thiophosgene (1.2 g, 11 mmol) and $CHCl_3$ (40 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation gave **69** (700 mg, 56%) as an oil: (lit. [Molina *et al* 1982] mp 111-112°C); Rf 0.7 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 4.88 (2 H, s, CH_2), 7.52 (2 H, d, J = 8.9 Hz, Ar 2,6-H_2), 8.25 (2 H, d, J = 8.9 Hz, Ar 3,5-H_2); IR ν_{max} (film) 1347 (NO_2), 1531 (NO_2), 2070 (N=C=S), 3079 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 195 (M + H).

N-(4-Nitrophenylmethyl)thiourea 70



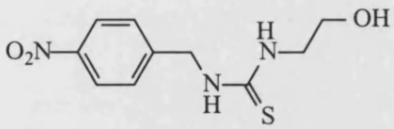
Ammonia was passed through **69** (250 mg, 1.3 mmol) in CH_2Cl_2 (40 ml) for 2 h. The mixture was then stirred for 3h at 0°C. Evaporation and chromatography (EtOAc) gave **70** (190 mg, 69%) as an oil: (lit. [M^{c}Kay *et al* 1958] oil) Rf 0.5; ^{1}H NMR ($CDCl_3$) δ 4.82 (2 H, br, CH_2), 5.82 (2 H, br, NH_2), 7.26 (1 H, s, NH), 7.50 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2), 8.21 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2); IR ν_{max} (film) 1159 (C=S), 1344 (NO_2), 1563 (NO_2), 3213 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 212.0490 (M + H) ($C_8H_{10}N_3O_2S$ requires 212.0494), 422 (2 M +H), 364 (M + mNBA), 196 (M – NH_2).

N-(4-Aminophenylmethyl)thiourea 71

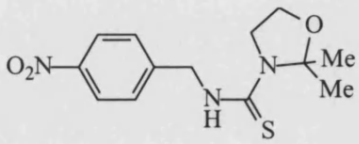


Compound **70** (150 mg, 0.7 mmol) was boiled under reflux with $SnCl_2.2H_2O$ (400 mg 1.8 mmol), in EtOH (9 ml) for 3 h. The solution was cooled to 0°C, made basic with 5% aq. $NaHCO_3$, extracted with EtOAc, washed with brine and dried. Evaporation and chromatography (EtOAc/acetic acid/hexane 10:1:1) gave **71** (120 mg 95 %) as pale orange crystals: mp >270°C; Rf 0.4 (EtOAc/acetic acid/hexane 10:1:1); ^{1}H NMR (CD_3OD) δ 4.54 (2 H, s, CH_2), 6.69 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.06 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2); IR ν_{max} (disc) 1179 (C=S), 3422 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* MS (FAB +ve ion) *m/z* 182.0746 (M + H) ($C_8H_{12}N_3S$ requires 182.0752), 164 (M – NH_3).

2,2-Dimethyl-3-(N-(4-nitrophenylmethyl)aminothiocarbonyl)tetrahydrooxazole 72 and **N-(2-hydroxyethyl)-N'-(4-nitrophenylmethyl)thiourea 73**



(73)



(72)

Compound **69** (350 mg, 2.3 mmol) in acetone (2.8 ml) was added dropwise during 30 min to 2-aminoethanol (140 mg, 2.3 mmol) in acetone (2.8 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **72** (90 mg, 13%) as an oil; Rf 0.6 (EtOAc); and **73** (200 mg, 34%) as an oil; Rf 0.4 (EtOAc)

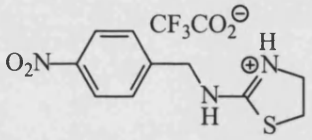
Compound 72

^{1}H NMR ($CDCl_3$) δ 1.79 (6 H, s, 2 × CH_3), 3.82 (2 H, m, oxazole 4-H_2), 4.05 (2 H, t, J = 6.2 Hz, oxazole 5-H_2), 5.03 (2 H, d, J = 5.4 Hz, CH_2Ar), 5.67 (1 H, br, NH), 7.49 (2 H, d, J = 8.8 Hz, Ar 2,6-H_2) 8.17 (2 H, d, J = 8.8 Hz, Ar 3,5-H_2); IR ν_{max} (film) 1143 (C=S), 1346 (NO_2), 1541 (NO_2), 3380 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 296.1063 (M + H) ($C_{13}H_{18}N_3O_3S$ requires 296.1069).

Compound 73

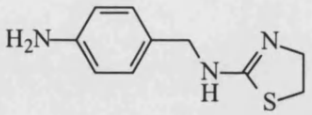
^{1}H NMR ($CDCl_3$) δ 3.62 (2 H, br, CH_2NH), 3.82 (2 H, t, J = 5.0 Hz, CH_2OH), 4.90 (2 H, d, J = 5.8 Hz, CH_2Ar), 6.54 (1 H, br, OH), 7.10 (1 H, br, NH), 7.26 (1 H, br NH), 7.50 (2 H, d, J = 8.9 Hz, Ar 2,6-H_2), 8.19 (2 H, d, J = 8.9 Hz, Ar 3,5-H_2); IR ν_{max} (film) 1160 (C=S), 1346 (NO_2), 1562 (NO_2), 3368 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 256.0755 (M + H) ($C_8H_{10}N_3O_2S$ requires 256.0756), 409 (M + mNBA).

2-(4-Nitrophenylmethylamino)-4,5-dihydrothiazole trifluoroacetate salt 74



Compound **73** (200 mg, 0.78 mmol) was stirred under reflux in trifluoroacetic acid (5 ml) for 15 h. Evaporation gave **74** (240 mg, quant.) as an hygroscopic gum: (lit. [Hirashima *et al* 1992] no mp given); Rf 0.6 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.54 (2 H, t, J = 7.8 Hz, thiazole 5-H_2), 4.04 (2 H, t, J = 7.8 Hz, thiazole 4-H_2), 4.59 (2 H, d, J = 5.1 Hz, CH_2Ar), 7.49 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2), 8.23 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2), IR ν_{max} (film) 1347 (NO_2), 1524 (NO_2), 1678 (C=N), 3173 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 238.0639 (M + H) ($C_{10}H_{12}N_3O_2S$ requires 238.0650), 222 (M – NH_2).

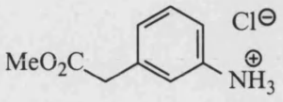
2-(4-Aminophenylmethylamino)-4,5-dihydrothiazole 75



Compound **74** (200 mg, 0.8 mmol) was boiled under reflux with $SnCl_2.2H_2O$ (460 mg 2.4 mmol) in EtOH (12 ml) for 90 min. The solution was cooled to 0°C, made basic with 5% aq. $NaHCO_3$, extracted with EtOAc, washed with brine and dried. Evaporation gave **75** (70 mg, 38%) as an oil; ^{1}H NMR ($CDCl_3$) δ 3.33 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 3.64 (2 H, br, NH_2), 4.02 (2 H, t, J = 7.4 Hz, thiazole 4-H_2), 4.34 (2 H, s, CH_2Ar), 6.64 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2), 7.11 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2); ^{13}C NMR 35.7 (CH_2S), 49.2 (CH_2Ar), 60.4 (CH_2N), 115.4 (Ar 3,5-C_2),

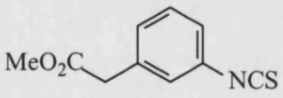
129.0 (Ar 1-C), 129.3 (Ar 2,6-C_2), 146.0 (Ar 4-C), 161.9 (C=N); IR ν_{max} (film) 1609 (C=N), 3324 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0911 (M + H) ($C_{10}H_{14}N_3S$ requires 208.0908), 361 (M + mNBA).

Methyl 3-aminophenylacetate hydrochloride 77



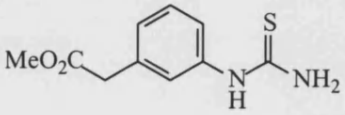
3-Aminophenylacetic acid **76** (2.0 g, 13.2 mmol) was stirred with MeOH (350 ml) and $SOCl_2$ (20 ml) for 4 d. Evaporation gave **77** (2.6 g, quant.) as an hygroscopic gum: (lit. [Jacobs *et al* 1917] mp 167-170°C) Rf 0.5 (EtOAc); ^1H NMR ($(CD_3)_2SO$) δ 3.60 (2 H, s, CH_2), 3.41 (3 H, br, NH_3^+), 3.74 (3 H, s, CH_3), 7.15 (3 H, m, Ar-H_3), 7.41 (1 H, m, Ar 5-H); MS (FAB +ve ion) *m/z* 166 (M + H), 319 (M + mNBA).

Methyl 3-isothiocyanatophenylacetate 78



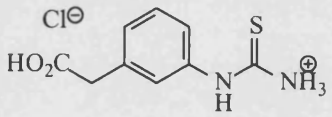
Compound **77** (2.0 g, 12 mmol), $CaCO_3$ (1.2 g, 12.0 mmol) water (5 ml), thiophosgene (2.8 g, 24 mmol) and $CHCl_3$ (80 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:1) gave **78** (1.9 g, 77%) as a pale yellow liquid; Rf 0.8 (EtOAc/hexane 1:1); ^1H NMR ($(CD_3)_2SO$) δ 3.61 (2 H, s, CH_2), 3.71 (3 H, s, CH_3), 7.26 (3 H, m, Ar-H_3), 7.30 (1 H, t, J = 7.8 Hz, Ar 5-H); IR ν_{max} (film) 1738 (C=O), 2119 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0432 (M + H) ($C_{10}H_{10}NO_2S$ requires 208.0432), 148 (M + H – NCS), 192 (M + H – CH_3).

Methyl 3-thioureidophenylacetate 79



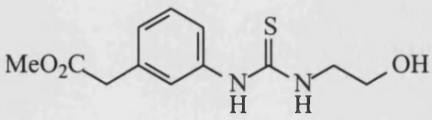
Ammonia was passed through **78** (150 mg, 0.7 mmol) in CH_2Cl_2 (20 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 1:1) gave **79** (60 mg, 99%) as yellow crystals: mp 131-133°C; Rf 0.2 (EtOAc/hexane 1:1); ^{1}H NMR ($(CD_3)_2SO$) δ 3.61 (3 H, s, CH_3), 3.65 (2 H, s, CH_2), 7.00 (1 H, d, J = 7.4 Hz, Ar 4-H), 7.23 (1 H, dd, J = 8.6, 7.4 Hz, Ar 5-H), 7.25 (1 H, s, Ar 2-H), 7.33 (1 H, d, J = 8.6 Hz, Ar 6-H), 7.35 (1 H, br, NH), 9.71 (1 H, s, NH); ^{13}C NMR ($(CD_3)_2SO$) δ 40.1 (CH_2), 51.8 (CH_3), 121.6 (C-2), 123.8 (C-4), 125.4 (C-6), 128.7 (C-5), 134.9 (C-1) 139.2 (C-3), 171.5 (C=S), 181.1 (C=O); IR ν_{max} (disc) 1160 (C=S), 1730 (C=O), 3409 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 225.0694 (M + H) ($C_{10}H_{13}N_2O_2S$ requires 225.0698), 377 (M + H + mNBA).

3-Thioureidophenylacetic acid hydrochloride 80



Compound **79** (50 mg, 0.2 mmol) was stirred in aq. HCl (1 M, 3 ml) for 9 d. Evaporation gave **80** (40 mg, 86%) as white crystals: mp 159-161°C (lit. [Richter *et al* 1974] mp 174-176°C); ^{1}H NMR (CD_3OD) δ 3.67 (2 H, s, CH_2), 7.15 (1 H, d, J = 7.4 Hz, Ar 4-H), 7.23 (1 H, d, J = 8.6 Hz, Ar 6-H), 7.25 (1 H, s, Ar 2-H), 7.35 (1 H, dd, J = 8.6, 7.4 Hz, Ar 5-H); IR ν_{max} (disc) 1157 (C=S), 1730 (C=O), 2500 (OH), 3337 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 211.0541 (M + H) ($C_9H_{11}N_2O_2S$ requires 211.0531), 364 (M + mNBA).

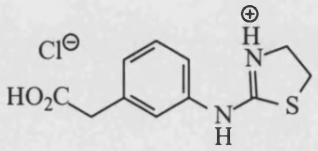
Methyl 3-(N'-(2-hydroxyethyl)thioureido)phenylacetate 81



Compound **78** (500 mg, 2.6 mmol) in acetone (3.2 ml) was added dropwise during 30 min to 2-aminoethanol (160 mg, 2.6 mmol) in acetone (3.2 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **81** (400 mg, 64%) as an oil; Rf 0.4 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.34 (2 H, m, CH_2N),

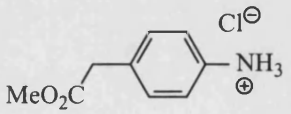
3.52 (2 H, br, CH_2Ar), 3.59 (3 H, s, CH_3), 3.63 (2 H, m, CH_2O), 4.70 (1 H, s, OH), 6.97 (1 H, d, J = 7.4 Hz, Ar 4-H) 7.24 (1 H, dd, J = 7.8, 6.3 Hz, Ar 5-H), 7.28 (1 H, s, Ar 2-H), 7.33 (1 H, d, J = 6.3 Hz, Ar 6-H), 7.69 (1 H, s, NH), 9.60 (1 H, br, NH); IR ν_{max} (film) 1061 (C=S), 1732 (C=O), 3293 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 269.0953 (M + H) ($C_{12}H_{17}N_2O_3S$ requires 269.0960), 537 (2 M + H).

3-(4,5-Dihydrothiazol-2-ylamino)phenylacetic acid hydrochloride 82



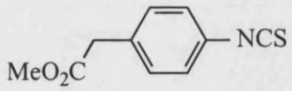
Compound **81** (200 mg, 0.8 mmol) was boiled under reflux for 40 h in aq. HCl (6 M, 10 ml). Evaporation gave **82** (200 mg, quant.) as an hygroscopic gum; Rf 0.2 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.55 (2 H, t, J = 7.6 Hz, thiazole 5-H_2), 3.93 (2 H, t, J = 7.6 Hz, thiazole 4-H_2), 5.75 (2 H, s, CH_2Ar), 7.25 (3 H, m, Ar-H_3), 7.44 (2 H, m, Ar-H + NH); ^{13}C NMR ($(CD_3)_2SO$) δ 31.0 (CH_2-S), 40.5 (CH_2-Ar), 48.7 (CH_2-N), 122.0 (C-4), 124.3 (C-2), 129.1 (C-6), 129.8 (C-5), 135.8 (C-1) 136.8 (C-3), 171.2 (C=N), 173.6 (C=O); IR ν_{max} (film) 1633 (C=N), 1714 (C=O), 2600 (OH), 3450 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 237.0698 (M + H) ($C_{11}H_{13}N_2O_2S$ requires 237.0698).

Methyl 4-aminophenylacetate hydrochloride 84



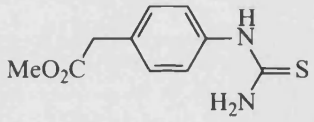
4-Aminophenylacetic acid **83** (2.0 g, 13.2 mmol) was stirred with MeOH (350 ml) and $SOCl_2$ (20 ml) for 4 d. Evaporation gave **84** (2.6 g quant.) as off-white crystals: mp 118-120°C (lit. [De Graw and Engstrom 1975] no mp given); Rf 0.5 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.47 (3 H, br, NH_3^+), 3.59 (3 H, s, CH_3), 3.68 (2 H, s, CH_2), 7.19 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2), 7.30 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2); MS (FAB +ve ion) *m/z* 166 (M + H), 121 (M – CO_2H).

Methyl 4-isothiocyanatophenylacetate 85



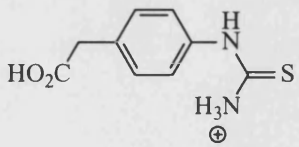
Compound **84** (1.0 g, 6.0 mmol), $CaCO_3$ (900 mg, 9.0 mmol), water (5 ml), thiophosgene (1.3 g, 12.0 mmol) and $CHCl_3$ (60 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation gave **85** (1.0 g, 83%) as an oil; (lit. [Shridhar *et al* 1985] oil); Rf 0.7 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.61 (3 H, s, CH_3), 3.73 (2 H, s, CH_2), 7.34 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2), 7.39 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2); IR ν_{max} (disc) 1738 (C=O), 2120 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 208 (M + H), 192 (M – Me).

Methyl 4-thioureidophenylacetate 86



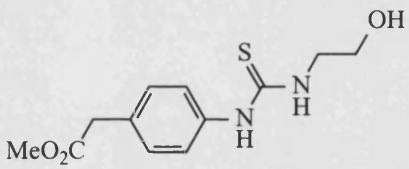
Ammonia was passed through **85** (150 mg, 0.7 mmol) in CH_2Cl_2 (50 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc) gave **86** (150 mg, 93%) as a white powder mp: 121-123°C; Rf 0.5 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.59 (3 H, s, CH_3), 3.62 (2 H, s, CH_2), 7.18 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.31 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.43 (2 H, br, NH_2), 9.65 (1 H, s, NH); IR ν_{max} (disc) 1718 (C=O), 3168 (OCH_3), 3341 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 225.0687 (M + H) ($C_{10}H_{13}N_2O_2S$ requires 225.0698), 378 (M + mNBA).

4-Thioureidophenylacetic acid hydrochloride 87



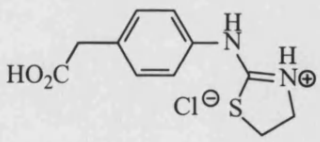
Compound **86** (50 mg, 0.2 mmol) was stirred for 16 h in aq. HCl (6 M, 5 ml). Evaporation gave **87** (60 mg, quant.) as white crystals: mp 198-200°C (lit. [Richter *et al* 1974] mp 200-203°C); ^{1}H NMR (CD_3OD) δ 3.65 (2 H, s, CH_2), 7.26-7.32 (4 H, m, Ar-H_4); IR ν_{max} (disc) 1181 (C=S), 1699 (C=O), 2900 (CO_2H), 3313 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 211.0541 (M + H) ($C_9H_{11}N_2O_2S$ requires 211.0531), 364 (M + mNBA).

Methyl 4-(N'-(2-hydroxyethyl)thioureido)phenylacetate 88



Compound **85** (200 mg, 1.0 mmol) in acetone (1.2 ml) was added dropwise during 30 min to 2-aminoethanol (60 mg, 1.0 mmol) in acetone (1.2 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **88** (70 mg, 27%) as yellow crystals: mp 53-55°C; Rf 0.3 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 2.35 (1 H, br, OH), 3.64 (2 H, s, CH_2Ar), 3.72 (3 H, s, CH_3), 3.80 (4 H, m, 2 × CH_2), 6.56 (1 H, br, NH), 7.20 (2 H, d, J = 7.8 Hz, Ar 3,5-H_2), 7.33 (2 H, d, J = 7.8 Hz, Ar 2,6-H_2), 7.94 (1 H, br, NH); IR ν_{max} (disc) 1169 (C=S), 1730 (C=O), 3325 (NH), 3480 (OH) cm^{-1}; MS (FAB +ve ion) *m/z* 269.0933 (M + H) ($C_{12}H_{16}N_2O_3S$ requires 269.0960), 537 (2 M +H), 422 (M + mNBA).

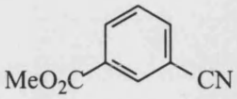
4-(4,5-Dihydrothiazol-2-ylamino)phenylacetic acid hydrochloride 89



Compound **88** (110 mg, 0.2 mmol) was stirred in aq HCl (6 M, 6 ml) for 16 h. Evaporation gave **89** (60 mg, quant.) as an hygroscopic gum; ^{1}H NMR (CD_3OD) δ 3.67 (4 H, m, thiazole 5-H_2 + CH_2Ar), 4.04 (2 H, m, thiazole 4-H_2), 7.30 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.43 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2); IR ν_{max} (disc) 1633 (C=N),

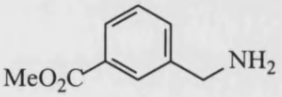
1736 (C=O), 2600 (CO_2H), 3423 (NH) cm^{-1}: MS (FAB +ve ion) *m/z* 237.0697 (M + H) ($C_{11}H_{13}N_2O_2S$ requires 237.0698).

Methyl 3-cyanobenzoate 91

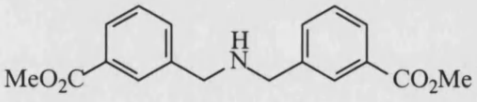


3-Cyanobenzoic acid **90** (2.0 g, 13.6 mmol) was stirred with MeOH (180 ml) and $SOCl_2$ (10 ml) for 4 d. Evaporation gave **91** (920 mg, 42%) as white crystals: mp 38-40°C (lit. [Tanaka *et al* 1998] oil); Rf 0.7 (EtOAc); ^1H NMR ($CDCl_3$) δ 3.96 (3 H, s, CH_3), 7.95 (1 H, t, *J* = 7.8 Hz, Ar 5-H), 7.84 (1 H, d, *J* = 7.8 Hz, Ar 4-H), 8.27 (1 H, d, *J* = 7.8 Hz, Ar 6-H), 8.34 (1 H, s, Ar 2-H); IR ν_{max} (disc) 1720 (C=O), 2228 (CN) cm^{-1}; MS (FAB +ve ion) *m/z* 162 (M + H), 315 (M + mNBA), 147 (M – CH_3).

Methyl 3-(aminomethyl)benzoate 92 and
Bis(3-methoxycarbonylphenylmethyl)amine 93



(92)



(93)

Compound **91** (900 mg, 5.6 mmol) in MeOH (30 ml) was treated with Pd/C (10%), and H_2 for 16 h. Evaporation and chromatography (EtOAc) gave **92** (330 mg 36%) as white crystals: mp 37-39°C (lit. [Ashton *et al* 1998] no mp given); Rf 0.1 (EtOAc) and **93** (150 mg, 8.6 %) as an oil (lit. [Ashton *et al* 1998] oil); Rf 0.5 (EtOAc);

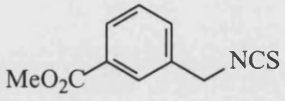
Compound 92

^1H NMR ($CDCl_3$) δ 3.92 (3 H, s, CH_3), 3.95 (2 H, s, CH_2), 7.40 (1 H, t, *J* = 7.8 Hz, Ar 5-H), 7.53 (1 H, d, *J* = 7.8 Hz, Ar 4-H), 7.92 (1 H, d, *J* = 7.8 Hz, Ar 6-H), 8.01 (1 H, s, Ar 2-H); IR ν_{max} (film) 1719 (C=O), 3453 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 166 (M + H), 121 (M – NH_2), 319 (M + mNBA).

Compound 93

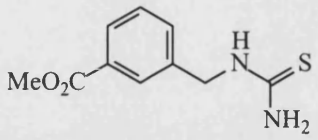
^{1}H NMR ($CDCl_3$) δ 3.84 (4 H, s, 2 × CH_2), 3.91 (6 H, s, 2 × CH_3), 7.40 (2 H, t, J = 7.8 Hz, 2 × Ar 5-H), 7.52 (2 H, d, J = 7.8 Hz, 2 × Ar 4-H), 7.93 (2 H, d, J = 7.8 Hz, 2 × Ar 6-H), 8.02 (2 H, s, 2 × Ar 2-H); IR ν_{max} (film) 1721 (C=O), 3336 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 314 (M + H), 467 (M + mNBA).

Methyl 3-(isothiocyanatomethyl)benzoate 94



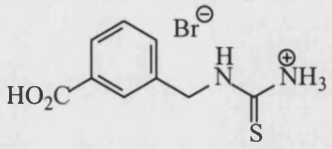
Compound **92** (270 mg, 1.6 mmol), $CaCO_3$ (160 mg, 1.6 mmol), water (3 ml), thiophosgene (360 mg, 3.2 mmol) and $CHCl_3$ (20 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc) gave **94** (170 mg, 51%) as an oil; Rf 0.7 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.94 (3 H, s, CH_3), 4.79 (2 H, s, CH_2), 7.50 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.54 (1 H, d, J = 7.8 Hz, Ar 4-H), 8.0 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.03 (1 H, s, Ar 2-H); IR ν_{max} (film) 1722 (C=O), 2101 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 208.0434 ($C_{10}H_{10}NO_2S$ requires 208.0432), 149 (M – NCS).

Methyl 3-(thioureidomethyl)benzoate 95



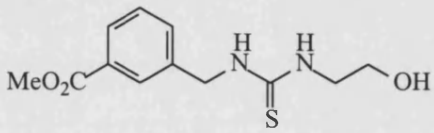
Ammonia was passed through **94** (60 mg, 0.3 mmol) in CH_2Cl_2 (20 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc) gave **95** (80 mg, quant.) as pale yellow crystals: mp 123-125°C; ^{1}H NMR (CD_3OD) δ 3.89 (3 H, s, CH_3), 4.88 (2 H, s, CH_2), 7.44 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.56 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.90 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.03 (1 H, s, Ar 2-H); IR ν_{max} (disc) 1202 (C=S), 1710 (C=O), 3418 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 225.0708 ($C_{10}H_{10}NO_2S$ requires 225.0698), 208 (M + H), 149 (M – NCS), 225 (M + H), 378 (M + mNBA), 449 (2 M + H), 211 (M – CH_3).

3-(Thioureidomethyl)benzoic acid hydrobromide 96



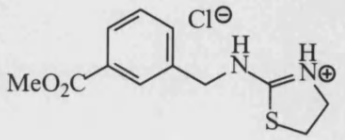
Compound **95** (80 mg, 0.4 mmol) was stirred in hydrobromic acid (50%, 5 ml) for 16 h. Evaporation gave **96** (70 mg, 98%) as an hygroscopic gum; ^{1}H NMR (CD_3OD) δ 3.89 (2 H, s, CH_2), 7.45 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.57 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.91 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.08 (1 H, s, Ar 2-H); IR ν_{max} (film) 1704 (C=O), 3298 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 225.0708 (M + H) ($C_{10}H_{13}N_2O_2S$) requires 225.0698).

Methyl 3-(N-(2-hydroxyethyl)thioureido)methyl)benzoate 97



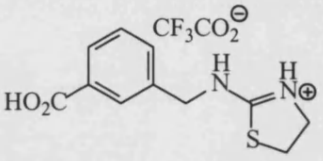
Compound **88** (80 mg, 0.4 mmol) in acetone (0.6 ml) was added dropwise during 30 min to 2-aminoethanol (20 mg, 0.4 mmol) in acetone (0.6 ml). The mixture was boiled under reflux for 2.5 h. Evaporation and chromatography (EtOAc) gave **97** (80 mg, 77%) as an oil; Rf 0.2 (EtOAc); ^{1}H NMR (CD_3OD) δ 3.60 (2 H, m, CH_2NH), 3.66 (2 H, m, CH_2OH), 3.89 (3 H, s, CH_3), 4.80 (2 H, s, CH_2Ar), 7.44 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.56 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.88 (1 H, d, J = 7.8 Hz, Ar 6-H), 7.96 (1 H, s, Ar 2-H); IR ν_{max} (film) 1197 (C=S), 1715 (C=O), 3355 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 269.0955 (M + H) ($C_{12}H_{17}N_2O_3S$ requires 269.0960), 537 (2 M + H).

Methyl 3-(4,5-dihydrothiazol-2-ylamino)benzoate hydrochloride 98



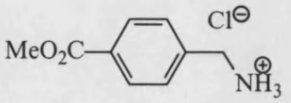
Compound **97** (70 mg, 0.3 mmol) was boiled under reflux for 40 h in aq. HCl (6 M, 5 ml). Evaporation gave crude 3-(4,5-dihydrothiazol-2-ylamino)methyl benzoic acid hydrochloride as an hygroscopic gum. This material (70 mg, 0.3 mmol) was stirred with MeOH (70 ml) and $SOCl_2$ (0.5 ml) for 4 d. Evaporation gave **98** (50 mg, 67%) as an oil; ^{1}H NMR ($CDCl_3$) δ 3.51 (2 H, m, thiazole 5-H_2), 3.92 (3 H, s, CH_3), 4.02 (2 H, m, thiazole 4-H_2), 4.54 (2 H, s, CH_2Ar), 7.47 (1 H, t, J = 7.4 Hz, Ar 5-H), 7.57 (1 H, d, J = 7.4 Hz, Ar 4-H), 7.95 (1 H, s, Ar 2-H), 7.99 (1 H, d, J = 7.4 Hz, Ar 6-H), 10.40 (1 H, br, NH), 10.93 (1 H, br, NH): IR ν_{max} (film) 1640 (C=N), 1718 (C=O), 3398 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 251.0859 (M + H) ($C_{12}H_{14}N_2O_2S$ requires 251.0854).

3-(4,5-Dihydrothiazol-2-ylaminomethyl)benzoic acid trifluoroacetate salt 99



Compound **98** (70 mg, 0.3 mmol) was stirred in aq. trifluoroacetic acid (50%, 5 ml) under reflux for 16 h. Evaporation gave **99** (70 mg, quant.) as an hygroscopic gum; ^{1}H NMR (CD_3OD) δ 3.65 (2 H, t, J = 7.8 Hz, thiazole 5-H_2), 4.04 (2 H, t, J = 7.8 Hz, thiazole 4-H_2), 4.60 (2 H, m, CH_2Ar), 7.54 (3 H, m, Ar-H_3), 8.02 (1 H, m, Ar-H); IR ν_{max} (disc) 1645 (C=N), 1696 (C=O), 2600 (CO_2H), 3433 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 237.0698 (M + H) ($C_{11}H_{13}N_2O_2S$ requires 237.0698).

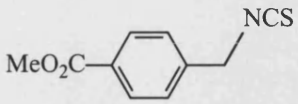
Methyl 4-(aminomethyl)benzoate hydrochloride 101



4-Aminomethylbenzoic acid **100** (2.0 g, 13.2 mmol), was stirred with MeOH (350 ml) and $SOCl_2$ (20 ml) for 4 d. Evaporation gave **101** (2.2 g, 99%) as white crystals: mp 153-155°C (lit. [Milkiewicz *et al* 2000] no mp given); Rf 0.5 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.84 (3 H, s, CH_3), 4.08 (2 H, s, CH_2), 7.64 (2 H, d, J = 8.2 Hz, Ar 3,5-

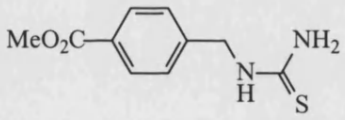
H_2), 7.96 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 8.62 (3 H, br, NH_3); MS (FAB +ve ion) *m/z* 319 (M + mNBA), 166 (M + H), 150 (M – NH_2).

Methyl 4-(isothiocyanatomethyl)benzoate 102



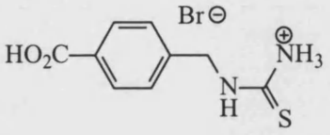
Compound **101** (1.0 g, 6.0 mmol), $CaCO_3$ (900 mg, 9.0 mmol), water (5 ml), thiophosgene (1.3 g, 12 mmol) and $CHCl_3$ (60 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation gave **102** (1.0 g, 83%) as a pale yellow liquid: (lit. [Emerson and Heimsch 1950] bp 138-152°C; Rf 0.6 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.88 (3 H, s, CH_3), 5.05 (2 H, s, CH_2), 7.49 (2 H, d, J = 8.4 Hz, Ar 3,5-H_2), 7.98 (2 H, d, J = 8.4 Hz, Ar 2,6-H_2); IR ν_{max} (film) 1715 (C=O), 2089 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 360 (M + mNBA), 208 (M + H), 192 (M – Me).

Methyl 4-(thioureidomethyl)benzoate 103



Ammonia was passed through compound **102** (150 mg, 0.7 mmol) in CH_2Cl_2 (20 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 1:1) gave **103** (60 mg, 99%) as white crystals: mp 131-133°C; Rf 0.2 (EtOAc/hexane 1:1); ^{1}H NMR ($(CD_3)_2SO$) δ 3.82 (3 H, s, CH_3), 4.70 (2 H, s, CH_2), 7.18 (2 H, br, NH_2), 7.38 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.90 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 8.06 (1 H, br NH); IR ν_{max} (disc) 1179 (C=S), 1711 (C=O), 3409 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 225.0690 (M + H) ($C_{10}H_{13}N_2O_2S$ requires 225.0698).

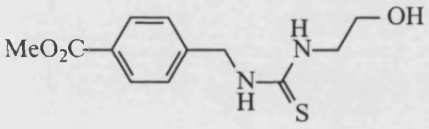
4-(Thioureidomethyl)benzoic acid hydrobromide 104



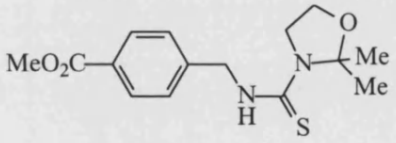
Compound **103** (40 mg, 0.2 mmol) was stirred in hydrobromic acid (50%, 8 ml) for 16 h. Evaporation gave **104** as an hygroscopic gum (40 mg, quant.); ^{1}H NMR (CD_3OD) δ 3.88 (2 H, s, CH_2), 7.41 (2 H, d, J = 8.3 Hz, Ar 3,5-H_2), 7.97 (2 H, d, J = 8.3 Hz, Ar 2,6-H_2); ν_{max} (disc) 1176 (C=S), 1705 (C=O), 3031 (CO_2H), 3382 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 211.0541 (M + H) ($C_9H_{11}N_2O_2S$ requires 211.0547).

2,2-Dimethyl-3-(N-(4-methoxycarbonylphenylmethyl)aminothiocarbonyl) tetrahydrooxazole 105 and

methyl 4-(N'-(2-hydroxyethyl)thioureidomethyl)benzoate 106



(106)



(105)

Compound **102** (500 mg, 2.4 mmol) in acetone (3.0 ml) was added dropwise during 30 min to 2-aminoethanol (160 mg, 2.4 mmol) in acetone (3.0 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **105** (280 mg, 43%) as white crystals: mp 105-107°C; Rf 0.3 (EtOAc); and **106** (400 mg, 64%) as buff crystals: mp 75-77°C; Rf 0.6 (EtOAc);

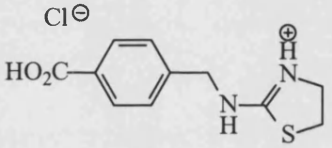
Compound 105

^{1}H NMR ($(CD_3)_2SO$) δ 1.69 (6 H, s, 2 × CH_3), 3.82 (3 H, s, OCH_3), 3.64 (2 H, t, J = 6.3 Hz, oxazole 4-H_2), 3.96 (2 H, t, J = 6.3 Hz, oxazole 5-H_2), 4.82 (2 H, d, J = 5.5 Hz, CH_2Ar), 7.39 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.88 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.84 (1 H, br, NH); IR ν_{max} (disc) 1199 (C=S), 1701 (C=O), 3359 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 309.1263 (M + H) ($C_{15}H_{21}N_2O_3S$ requires 309.1273).

Compound 106

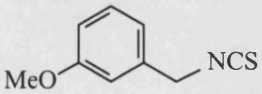
^{1}H NMR ($(CD_3)_2SO$) δ 3.47 (4 H, m, 2 × CH_2), 3.82 (3 H, s, CH_3), 4.74 (3 H, m, CH_2Ar + OH), 7.38 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.89 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 7.58 (1 H, br, NH), 8.01 (1 H, br, NH); IR ν_{max} (film) 1194 (C=S), 1714 (C=O), 3351 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 269.0961 (M + H) ($C_{12}H_{16}N_2O_3S$ requires 269.0960).

4-(4,5-Dihydrothiazol-2-ylaminomethyl)benzoic acid hydrochloride 107



Compound **106** (70 mg, 0.3 mmol) was boiled under reflux for 40 h in aq. HCl (6 M, 5 ml). Evaporation gave **107** (200 mg, quant.) as yellow crystals: mp 138-140°C; Rf 0.2 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.57 (2 H, t, J = 7.4 Hz, thiazole 5-H_2), 3.92 (2 H, t, J = 7.4 Hz, thiazole 4-H_2), 4.73 (2 H, s, CH_2Ar), 7.46 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.95 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2), 10.66 (1 H, br, NH), 13.05 (1 H, br, NH); IR ν_{max} (film) 1656 (C=N), 1697 (C=O), 2600 (OH), 3430 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 237.0688 (M + H) ($C_{11}H_{13}N_2O_2S$ requires 237.0698), 390 (M + mNBA).

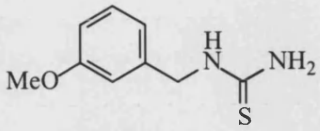
3-Methoxyphenylmethylisothiocyanate 109



3-Methoxybenzylamine **108** (1.5 g, 11 mmol), $CaCO_3$ (1.0 g, 1.0 mmol), water (8 ml), thiophosgene (1.1 g, 9.2 mmol) and $CHCl_3$ (80 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:1) gave **109** (800 mg, 40%) as an oil: (lit. [Ettlinger and Lundeen 1956] oil); Rf 0.6 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 3.82 (3 H, s, CH_3), 4.68 (2 H, s, CH_2), 6.87 (3 H, m, Ar 2,4,6-H_3), 7.30 (1 H, t, J =

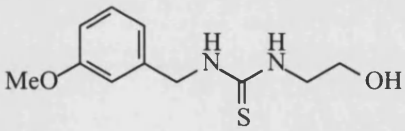
7.8 Hz, Ar 5-H); IR ν_{max} (film) 2094 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 180.0460 (M + H) ($C_9H_{10}NOS$ requires 180.0483), 121 (M – NCS).

N-(3-Methoxyphenylmethyl)thiourea 110



Ammonia was passed through compound **109** (300 mg, 1.7 mmol) in CH_2Cl_2 (50 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc) gave **110** (340 mg, quant.) as an oil: (lit. [Ettlinger and Lundeen 1956] mp 101-103°C) Rf 0.5 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.80 (3 H, s, CH_3), 4.20 (1 H, br, NH), 4.77 (2 H, m, CH_2), 5.83 (2 H, s, NH_2), 6.86 (3 H, m, Ar 2,4,6-H_3), 7.27 (1 H, m, Ar 5-H); IR ν_{max} (film) 1046 (C=S), 2835 (OCH_3), 3273 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 197.0751 (M + H) ($C_9H_{13}N_2OS$ requires 197.0749), 121 (M – $NHCSNH_2$).

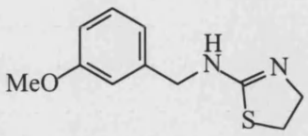
N-(2-Hydroxyethyl)-N'-(3-methoxyphenylmethyl)thiourea 111



Compound **109** (100 mg, 0.5 mmol) in acetone (0.6 ml) was added dropwise during 30 min to 2-aminoethanol (30 mg, 0.5 mmol) in acetone (0.6 ml). The mixture was boiled under reflux for 4 h. Evaporation and chromatography (EtOAc) gave **111** (100 mg, 85%) as an oil; Rf 0.3 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.36 (4 H, m, 2 × CH_2), 3.67 (3 H, s, CH_3), 4.55 (2 H, m, CH_2Ar), 4.78 (1 H, s, OH), 6.80 (1 H, dd, *J* = 7.4, 2.1 Hz, Ar 6-H), 6.86 (1 H, dd, *J* = 7.4, 2.1 Hz, Ar 4-H), 6.85 (1 H, s, Ar 2-H), 7.23 (1 H, t, *J* = 7.4 Hz, Ar 5-H), 7.52 (1 H, br, NH), 7.92 (1 H, br, NH); ^{13}C NMR ($(CD_3)_2SO$) δ 46.7 (CH_2), 48.5 (CH_2), 55.3 (CH_2-Ar), 112.9 (C-2) 113.2 (C-3), 119.7 (C-5,6), 129.7 (C-1,3), 159.6 (C=S); IR ν_{max} (film) 1167 (C=S), 3292 (NH + OH)

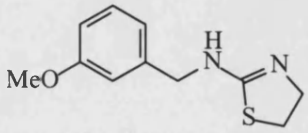
cm^{-1}; MS (FAB +ve ion) *m/z* 241.1009 (M + H) ($C_{11}H_{17}N_2O_2S$ requires 241.1011), 393 (M + mNBA).

2-(3-Methoxyphenylmethylamino)-4,5 dihydrothiazole 112



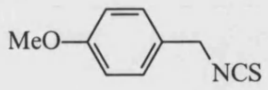
Compound **110** was stirred in trifluoroacetic acid (5 ml) for 2 h. Evaporation and chromatography (EtOAc/MeOH 5:1) gave **112** (200 mg, 75%) as an oil; Rf 0.2 (EtOAc/MeOH 5:1); ^{1}H NMR ($CDCl_3$) δ 3.46 (2 H, t, *J* = 7.6 Hz, thiazole 5-H_2), 3.82 (3 H, s, CH_3), 3.95 (2 H, t, *J* = 7.6 Hz, thiazole 4-H_2), 4.43 (2 H, d, *J* = 5.6 Hz, CH_2Ar), 6.87 (3 H, m, Ar 2,4,6-H_3), 7.28 (1 H, m, Ar 5-H), 12.25 (1 H, s, NH), 12.36 (1 H, s, NH); ^{13}C NMR ($CDCl_3$) δ 31.1 (CH_2S), 48.7 (CH_2N), 51.3 (CH_2Ar), 55.2 (CH_3), 112.9 (Ar 2-C), 114.0 (Ar 4-C), 119.6 (Ar 6-C), 130.0 (Ar 5-C), 136.5 (Ar 1-C), 160.0 (Ar 3-C), 174.6 (C=N); IR ν_{max} (film) 1681 (C=N), 3200 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 223.0890 (M + H) ($C_{11}H_{15}N_2OS$ requires 223.0905).

2-(3-Methoxyphenylmethylamino)-4,5-dihydrothiazole 112



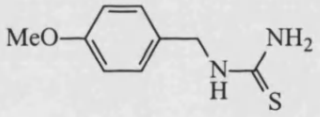
Compound **108** was heated with **20** (320 mg, 2.4 mmol) at 180°C for 4 h. Evaporation and chromatography (EtOAc/MeOH 5:1) gave **112** (163 mg, 30%) as white crystals: mp 94-96°C; Rf 0.2 (EtOAc/MeOH 5:1); ^{1}H NMR ($CDCl_3$) δ 3.59 (2 H, t, *J* = 7.4 Hz, thiazole 5-H_2), 3.80 (3 H, s, CH_3), 4.04 (2 H, t, *J* = 7.4 Hz, thiazole 4-H_2), 4.40 (2 H, s, CH_2Ar), 4.58 (1 H, br, NH), 6.87 (3 H, m, Ar 2,4,6-H_3), 7.25 (1 H, m, Ar 5-H); IR ν_{max} (disc) 1653 (C=N), 3420 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 223.0902 (M + H) ($C_{11}H_{15}N_2OS$ requires 223.0905).

4-Methoxyphenylmethylisothiocyanate 113



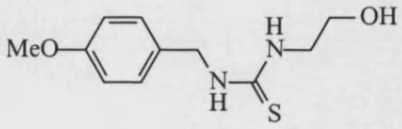
Compound **19** (1.0 g, 7.3 mmol), $CaCO_3$ (0.7 g, 0.7 mmol), water (5 ml), thiophosgene (1.6 g, 15 mmol) and $CHCl_3$ (50 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:1) gave **113** (1.0 g 80%) as a pale yellow liquid: (lit. [Buckley and Oppenheimer 1994] liquid); Rf 0.7 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 3.81 (3 H, s, CH_3), 4.63 (2 H, s, CH_2), 6.93 (2 H, d, J = 8.2 Hz, Ar 3,5-H_2), 7.22 (2 H, d, J = 8.2 Hz, Ar 2,6-H_2); IR ν_{max} (film) 2087 (N=C=S) cm^{-1}; MS (FAB +ve ion) *m/z* 179 (M), 121 (M-NCS).

N-(4-Methoxyphenylmethyl)thiourea 114



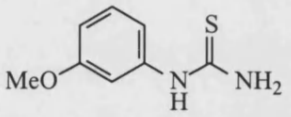
Ammonia was passed through compound **113** (600 mg, 3.2 mmol) in CH_2Cl_2 (50 ml) for 30 min. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/hexane 1:1) gave **114** (600 mg, 99%) as white crystals: mp 124-126°C (lit. [Hűter 1947] mp 135°C); ^{1}H NMR ($CDCl_3$) δ 3.87 (3 H, s, CH_3), 4.38 (2 H, br, CH_2), 5.75 (2 H, br, NH_2), 6.88 (1 H, br, NH), 6.90 (2 H, d, J = 8.4 Hz, Ar 3,5-H_2), 7.23 (2 H, d, J = 8.4 Hz, Ar 2,6-H_2); IR ν_{max} (disc) 1177 (C=S), 2800 (OCH_3), 3165 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 197.0749 (M + H) ($C_9H_{13}N_2OS$ requires 197.0751), 393 (2 M + H), 350 (M + mNBA), 121 (M – CH_3N_2S); Found C, 54.80: H, 6.13; N, 14.14: $C_9H_{12}N_2OS$ requires C, 54.30; H, 6.11; N, 14.10%.

N-(2-Hydroxyethyl)-N'-(4-methoxyphenylmethyl)thiourea 115



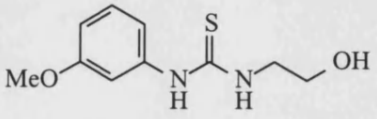
Compound **113** (400 mg, 2.2 mmol) in acetone (2.7 ml) was added dropwise during 30 min to 2-aminoethanol (134 mg, 2.2 mmol) in acetone (2.7 ml). The mixture was boiled under reflux for 2 h. Evaporation and chromatography (EtOAc) gave **115** (250 mg, 47%) as an oil; Rf 0.3 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.49 (3 H, s, CH_3), 3.66 (2 H, br, CH_2NH), 3.81 (2 H, m, CH_2OH), 4.58 (2 H, s, CH_2Ar), 6.48 (1 H, br, NH), 6.74 (1 H, br, NH), 6.87 (2 H, d, J = 8.6 Hz, Ar 3,5-H_2), 7.25 (2 H, d, J = 8.6 Hz, Ar 2,6-H_2); IR ν_{max} (film) 1171 (C=S), 3336 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 241.1011 (M + H) ($C_{11}H_{17}N_2O_2S$ requires 241.1018), 394 (M + mNBA).

N-(3-Methoxyphenyl)thiourea 117



Ammonia was passed through 3-methoxyphenylisothiocyanate **116** (500 mg, 3.0 mmol) in CH_2Cl_2 (75 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation gave **117** (600 mg, quant.) as white crystals: mp 160-162°C (lit. [Rasmussen *et al* 1988] mp 109°C); Rf 0.5 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.73 (3 H, s, CH_3), 6.67 (1 H, d, J = 8.2 Hz, Ar 4-H), 6.90 (1 H, d, J = 7.6 Hz, Ar 6-H), 7.10 (1 H, s, Ar 2-H), 7.23 (1 H, dd, J = 8.2, 7.6 Hz, Ar 5-H), 7.53 (2 H, br, NH_2) 9.62 (1 H, s, NH); ^{13}C NMR ($(CD_3)_2SO$) δ 55.1 (CH_3), 108.6 (C-2) 110.0 (C-6), 115.0 (C-4), 129.6 (C-5), 140.3 (C-1), 159.3 (C-3), 180.9 (C=S); IR ν_{max} (disc) 1166 (C=S), 3149 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 183.0598 (M + H) ($C_8H_{11}N_2OS$ requires 183.0592), 336 (M + mNBA), 365 (2 M +H); Found C, 52.50: H, 5.53; N, 15.40; $C_8H_{10}N_2OS$ requires C, 52.72; H, 5.53; N, 15.37%.

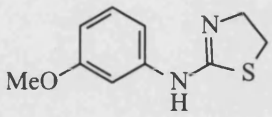
N-(2-Hydroxyethyl)-N'-(3-methoxyphenyl)thiourea 118



Compound **116** (500 mg, 3.0 mmol) in acetone (3.5 ml) was added dropwise during 30 min to 2-aminoethanol (183 mg, 3.0 mmol) in acetone (3.5 ml). The mixture was

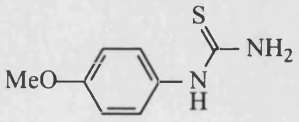
boiled under reflux for 2 h. Evaporation gave **118** (570 mg, 84%) as white crystals: mp 129-131°C; Rf 0.4 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.52 (4 H, m, 2 × CH_2), 3.70 (3 H, s, CH_3), 4.80 (1 H, br, OH), 6.65 (1 H, d, J = 7.8 Hz, Ar 6-H), 6.90 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.20 (2 H, m, Ar 2,5-H_2), 7.71 (1 H, br, NH), 9.41 (1 H, s, NH); IR ν_{max} (disc) 1149 (C=S), 2900 (OCH_3), 3186 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 227.0846 (M + H) ($C_{10}H_{15}N_2O_2S$ requires 227.0854), 452 (2M + H); Found C, 53.4: H, 6.28; N, 12.36; $C_{10}H_{14}N_2O_2S$ requires C, 53.08; H, 6.24; N, 12.38%.

2-(3-Methoxyphenylamino)-4,5-dihydrothiazole 119



Compound **118** was stirred in trifluoroacetic acid (5 ml) for 3 h. Evaporation and chromatography (EtOAc) gave **119** (130 mg, 70%) as buff crystals: mp 80-82°C; Rf 0.2 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 4.17 (3 H, s, CH_3), 3.92 (2 H, t, J = 7.6 Hz, thiazole 5-H_2), 4.35 (2 H, t, J = 7.6 Hz, thiazole 4-H_2) 7.24 (1 H, d, J = 8.2 Hz, Ar 4-H), 7.34 (1 H, d, J = 8.2 Hz, Ar 6-H), 7.41 (1 H, s, NH), 7.73 (1 H, t, J = 8.2 Hz, Ar 5-H), 7.74 (1 H, s, Ar 2-H); IR ν_{max} (disc) 1674 (C=N), 2850 (OCH_3), 3238 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 209.0749 (M + H) ($C_{10}H_{13}N_2OS$ requires 209.0743), 362 (M + mNBA).

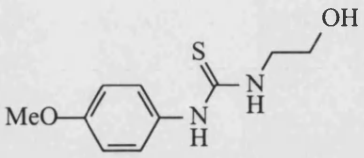
N-(4-Methoxyphenyl)thiourea 121



Ammonia was passed through 4-methoxyphenylisothiocyanate **120** (500 mg, 3.0 mmol) in CH_2Cl_2 (75 ml) for 1 h. The mixture was then stirred for 3 h at 0°C; Evaporation gave **121** (340 mg, quant.) as white crystals: mp 198-200°C (lit. [Rasmussen *et al* 1988] mp 155-157°C); Rf 0.5 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.71 (3 H, s, CH_3), 6.89 (2 H, d, J = 8.8 Hz, Ar 3,5-H_2), 7.21 (2 H, d, J = 8.8 Hz, Ar 2,6-

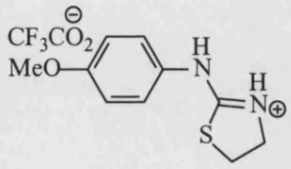
H_2), 7.23 (2 H, br, NH_2), 9.47 (1 H, s, NH); IR ν_{max} (disc) 1171 (C=S), 2838 (OCH_3), 3154 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 183.0592 (M + H) ($C_8H_{10}N_2OS$ requires 183.0592), 335 (M + mNBA).

N-(2-Hydroxyethyl)-N'-(4-methoxyphenyl)thiourea 122



Compound **120** (500 mg, 3.0 mmol) in acetone (3.5 ml) was added dropwise during 30 min to 2-aminoethanol (183 mg, 3.0 mmol) in acetone (3.5 ml). The mixture was boiled under reflux for 90 min. Evaporation and chromatography (EtOAc) gave **122** (600 mg, 88%) as buff crystals: mp 145-147°C (lit. [Adock and Lawson 1965] 146-147°C); Rf 0.5 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.35 (3 H, s, CH_3), 3.73 (4 H, m, 2 × CH_2), 4.79 (1 H, br, OH), 6.88 (2 H, d, *J* = 8.6 Hz, Ar 3,5-H_2), 7.24 (2 H, d, *J* = 8.6 Hz, Ar 2,6-H_2), 7.46 (1 H, br, NH), 9.41 (1 H, s, NH); IR ν_{max} (disc) 1165 (C=S), 2835 (OCH_3), 3189 (NH), 3646 (OH) cm^{-1}; MS (FAB +ve ion) *m/z* 227.0850 (M + H) ($C_{10}H_{15}N_2O_2S$ requires 227.0854), 380 (M + mNBA); Found C, 53.0: H, 6.17; N, 12.2: $C_{11}H_{14}N_2O_2S$ requires C, 53.08; H, 6.24; N, 12.38%.

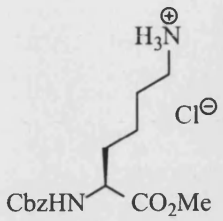
2-(4-Methoxyphenylamino)-4,5-dihydrothiazole trifluoroacetate salt 123



Compound **122** (200 mg, 0.88 mmol) was stirred in trifluoroacetic acid (5 ml) under reflux for 15 h. Evaporation gave **123** (220 mg, quant.) as white crystals: mp 101-103°C (lit. [Tisler *et al* 1958] mp 127.5°C for free base); Rf 0.6 (EtOAc/MeOH 1:1); ^{1}H NMR ($(CD_3)_2SO$) δ 2.29 (3 H, s, CH_3), 3.60 (2 H, m, thiazole 5-H_2) 3.75 (2 H, m, thiazole 4-H_2), 7.02 (2 H, d, *J* = 6.9 Hz, Ar 3,5-H_2), 7.25 (2 H, d, *J* = 6.9 Hz, Ar 2,6-H_2), 7.97 (1 H, br, NH); IR ν_{max} (disc) 1674 (C=N), 2750 (OCH_3), 3409 (NH) cm^{-1};

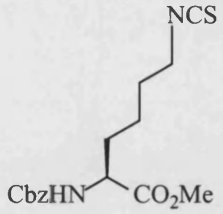
MS (FAB +ve ion) *m/z* 209.0758 (M + H) ($C_{10}H_{13}N_2OS$ requires 209.0749), 362 (M + mNBA).

N^{α}-(Phenylmethoxycarbonyl)-L-lysine methyl ester hydrochloride 125



Cbz-Lys-OH **124** (2.1 g, 7.5 mmol) was stirred with MeOH (100 ml) and $SOCl_2$ (11 ml) for 4 d. Evaporation gave **125** (1.5 g, 65%) as an hygroscopic gum: (lit. [Ranganathan *et al* 1993] mp 113-115°C for free base); Rf 0.5 (EtOAc/MeOH 5:1); ^{1}H NMR ($(CD_3)_2SO$) δ 1.35 (2 H, m, Lys γ-H_2), 1.45-1.80 (4 H, m, Lys, β,δ-H_4), 2.75 (2 H, m, Lys ε-H_2), 3.68 (3 H, s, CH_3), 4.00 (1 H, m, Lys α-H), 5.03 (2 H, m, $PhCH_2$), 7.38-7.45 (5 H, m, Ph-H_5), 7.75 (1 H, d, J = 7.8 Hz, NH), 7.99 (1 H, br, NH_3^+); $[\alpha]_D^{20}$ = -10.6° (c = 1.6 mg ml^{-1}, MeOH); MS (FAB +ve ion) *m/z* 295.1662 (M + H) ($C_{15}H_{23}N_2O_4$ requires 295.1658), 264 (M - OCH_3).

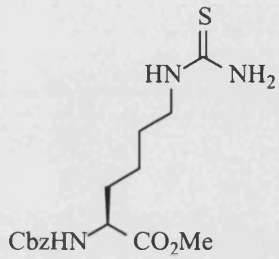
Methyl *S*-6-isothiocyanato-2-(phenylmethoxycarbonylamino)hexanoate 126



Compound **125** (1.0 g, 3.4 mmol), $CaCO_3$ (400 mg, 4.4 mmol), water (5 ml), thiophosgene (780 mg, 6.8 mmol) and $CHCl_3$ (40 ml) were stirred vigorously for 16 h. The mixture was extracted with $CHCl_3$ and dried. Evaporation gave **126** (600 mg, 52%) as an oil; Rf 0.5 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.48 (2 H, m, 4-H_2), 1.66 (4 H, m, 3,5-H_4), 3.51 (2 H, t, J = 6.2 Hz, 6-H_2), 3.76 (3 H, s, CH_3), 4.40 (1 H, m, 1-H), 5.03 (2 H, s, CH_2Ar), 5.35 (1 H, d, J = 7.8 Hz, NH), 7.36-7.38 (5 H, m, Ar-H_5); ^{13}C NMR ($(CD_3)_2SO$) δ 22.6 (C-4), 28.8 (C-3), 28.8 (C-5), 44.8 (C-6), 51.9

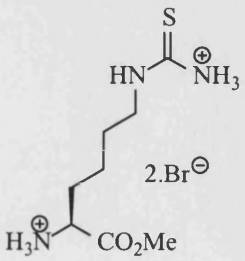
(CH_3), 53.6 (C-2), 126.9 (CH_2-Ar), 126.9 (Ar-4), 127.7 (Ar-2), 128.2 (Ar-3), 128.5 (Ar-5), 128.7 (Ar-6), 136.8 (Ar-1), 155.9 (C=S), 172.5, 173.5 (2 × C=O); IR ν_{max} (film) 1721 (C=O), 2109 (N=C=S), 3348 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 337.1218 (M + H) ($C_{16}H_{21}N_2O_4S$ requires 337.1222), 673 (2 M + H).

N^{ε}-Aminothiocarbonyl-N^{α}-phenylmethoxycarbonyl-L-lysine methyl ester 127



Ammonia was passed through **126** (300 mg, 0.9 mmol) in CH_2Cl_2 (30 ml) for 1 h. The mixture was then stirred for 3 h at 0°C. Evaporation and chromatography (EtOAc/MeOH 5:1) gave **127** (180 mg, 59%) as a yellow oil; Rf 0.3 (EtOAc/MeOH 5:1); ^{1}H NMR ($(CD_3)_2SO$) δ 1.40 (2 H, m, Lys γ-H_2), 1.65 (4 H, m, Lys, β,δ-H_4), 3.05 (1 H, m) and 3.56 (1 H, m) (Lys ε-H_2), 3.75 (3 H, s, CH_3), 4.34 (1 H, m, Lys α-H), 5.03 (2 H, s, CH_2Ar), 6.89 (1 H, br NH), 7.34-7.37 (7 H, m, Ar-H_5 + NH_2) 7.74 (1 H, s, NH); IR ν_{max} (film) 1159 (C=S), 3200 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 354.1497 (M + H) ($C_{16}H_{24}N_3O_4S$ requires 354.1488).

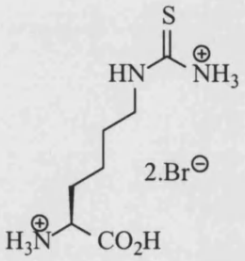
N^{ε}-Aminothiocarbonyl-L-lysine methyl ester dihydrobromide 128



Compound **127** (100 mg, 0.3 mmol) was stirred in HBr in HOAc (17.5%, 4 ml) for 15 h. Dry Et_2O (10 ml) was added, the mixture was stirred for 5 min and then Et_2O was decanted; this was repeated 8 times. Drying gave **128** (78 mg, quant.) as a

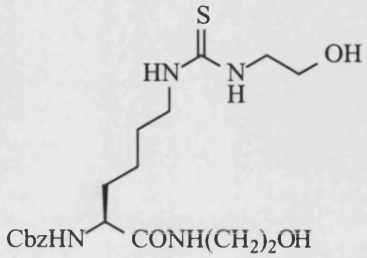
colourless hygroscopic gum; Rf 0.4 (MeOH); ^{1}H NMR ($(CD_3)_2SO$) δ 1.43-1.90 (6 H, m, Lys β,γ,δ-H_6), 3.29 (2 H, m, Lys ε-H_2), 3.67 (3 H, s, CH_3), 4.01 (1 H, m, Lys α-H), 7.52 (1 H, br NH), 8.34 (1 H, s, NH); IR ν_{max} (film) 1240 (C=S), 1742 (C=O), 3030 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 220.1119 (M + H) ($C_8H_{18}N_3O_2S$ requires 220.1120).

N$^{\varepsilon}$-Aminothiocarbonyl-L-lysine dihydrobromide 129



Compound **128** (50 mg, 0.2 mmol) was stirred in hydrobromic acid (5 ml) for 16 h. Evaporation gave **129** (60 mg, quant) as a colourless hygroscopic gum; ^{1}H NMR (CD_3OD) δ 1.5-2.0 (6 H, m, Lys β,γ,δ-H_6), 2.96 (2 H, t, J = 7.8 Hz, Lys ε-H_2), 4.01 (1 H, t, J = 6.6 Hz, Lys α-H); ^{13}C NMR δ 22.3 (C-γ), 27.4 (C-β), 30.1 (C-δ), 43.9 (C-ε), 52.7 (C-α), 128.8 (C=O), 170.4 (C=S); IR ν_{max} (film) 1194 (C=S), 1739 (C=O), 3429 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 206.0956 (M + H) ($C_7H_{16}N_3O_2S$ requires 206.0963).

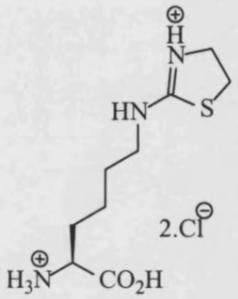
N$^{\alpha}$-(Phenylmethoxycarbonyl)-N$^{\varepsilon}$-(N-(2-hydroxyethyl)aminothiocarbonyl)lysine N-(2-hydroxyethyl)amide 130



Compound **125** (500 mg, 1.5 mmol) in acetone (2 ml) was added dropwise during 30 min to 2-aminoethanol (82 mg, 1.4 mmol) in acetone (2 ml). The mixture was boiled

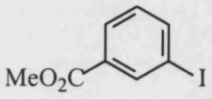
under reflux for 4 h. Evaporation and chromatography (EtOAc/MeOH 5:1) gave **130** (340 mg, 53%) as an oil; Rf 0.6 (EtOAc/MeOH 5:1); ^{1}H NMR ($(CD_3)_2SO$) δ 1.43 (6 H, m, Lys β,γ,δ-H_6), 3.15 (2 H, m, Lys ε-H_2), 3.35 (4 H, m) and 3.44 (2 H, m) and 3.94 (2 H, m) (2 × CH_2CH_2O), 3.94 (1 H, m, NHCbz), 4.25 (1 H, t, J = 7.4 Hz, NH), 4.67 (1 H, t, J = 5.5 Hz, Lys α-H), 5.01 (2 H, s, $PhCH_2$), 7.10 (1 H, br, NH), 7.34-7.40 (5 H, m, Ar-H_5), 7.87 (1 H, br, NH); IR ν_{max} (film) 1148 (C=S), 1711 (C=O), 3370 (NH) cm^{-1}; MS (FAB +ve) *m/z* 875 (2 M + Na), 853 (2 M + H), 449 (M + Na), 427 (M + H), 91 (Bn).

N^{ε}-(4,5-Dihydrothiazol-2-yl)lysine dihydrochloride 131



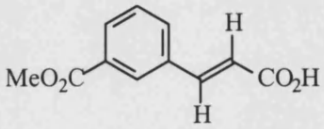
Compound **130** (330 mg, 0.91 mmol) was boiled under reflux for 36 h in aq. HCl (6 M, 4 ml). Evaporation gave **131** (160 mg, 76%) as an hygroscopic gum; ^{1}H NMR ($(CD_3)_2SO$) δ 1.44 (6 H, m, Lys β,γ,δ-H_6), 2.80 (2 H, m, Lys ε-H_2), 3.35 (2 H, m, thiazole 5-H_2), 3.49 (1 H, m, Lys α-H), 3.58 (2 H, m, thiazole 4-H_2), 8.10 (3 H, br, N^+H_3) 8.50 (2 H, br, 2 × NH); ^{13}C NMR ($(CD_3)_2SO$) δ 21.6 (C-δ), 29.5 (C-δ), 30.9 (C-β), 38.6 (CH_2-S), 44.8 (C-ε), 51.8 (C-α), 57.6 (CH_2-N), 169.6 (C=N), 171.0 (C=O); IR ν_{max} (film) 1651 (C=N), 1750 (C=O), 2600 (CO_2H), 3413 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 232.1115 (M + H) ($C_9H_{18}N_3O_2S$ requires 232.1120), 215 (M – NH_3).

Methyl 3-iodobenzoate 133



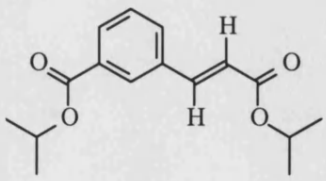
3-Iodobenzoic acid **132** (30 g, 120 mmol), was stirred with MeOH (600 ml) and $SOCl_2$ (90 ml) for 4 d. Evaporation gave **133** (31 g, 98%) as white crystals: mp 47-49°C (lit. [Chaikovski *et al* 2000] mp: 52-54°C); Rf 0.7 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 3.92 (3 H, s, CH_3), 7.17 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.88 (1 H, dd, J = 1.0, 7.8 Hz, Ar 6-H), 7.98 (1 H, dd, J = 1.0, 7.8 Hz, Ar 4-H), 8.38 (1 H, s, Ar 2-H); MS (EI +ve ion) *m/z* 262 (M).

Methyl 3-(2-carboxyethenyl)benzoate 134



Compound **133** (1 g, 4 mmol), propenoic acid (360 mg, 5 mmol), palladium(II) acetate (85 mg, 0.4 mmol), Et_3N (250 mg, 2.5 mmol) in propanenitrile (3 ml) were boiled under reflux for 1 h. aq. HCl (100 ml, 2 M) was added to the cooled mixture. The precipitate, in hot ethanol, was filtered and the filtrate was allowed to cool slowly. The solid was collected and dried to give **134** (850 mg, quant.) as off-white crystals: mp: 145-147°C; Rf 0.4 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.87 (3 H, s, CH_3), 6.61 (1 H, d, J = 16.0 Hz, $CHCO_2H$), 7.56 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.66 (1 H, d, J = 16.0 Hz, CH-Ar), 7.97 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.98 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.18 (1 H, s, Ar 2-H); MS (FAB +ve ion) *m/z* 207.0659 ($C_{11}H_{11}O_4$ requires 207.0657), 162 (M – CO_2H).

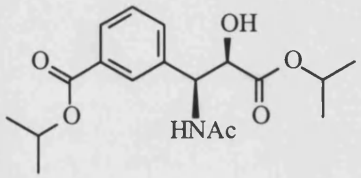
Prop-2-yl 3-(3-(prop-2-yloxy(carbonyl)phenyl)propanoate 135



Compound **134** (200 mg, 1.0 mmol) in propan-2-ol (20 ml) and conc. H_2SO_4 (1 ml) was heated under reflux for 24 h. The mixture was cooled, poured onto ice and then extracted with diethyl ether. The extracts were dried. Evaporation and

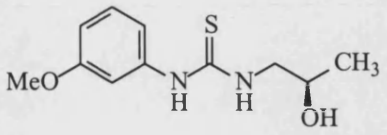
chromatography gave **135** (140 mg, 50%) as an oil; Rf 0.8 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.32 (6 H, d, J = 6.3 Hz, 2 × CH_3), 1.38 (6 H, d, J = 6.3 Hz, 2 × CH_3), 1.39 (3 H, s, CH_3), 5.16 (1 H, m, CH), 5.24 (1 H, m, CH), 6.48 (1 H, d, J = 16.1 Hz, $CHCO_2H$), 7.46 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.68 (1 H, d, J = 7.8 Hz, Ar 4-H), 7.69 (1 H, d, J = 16.0 Hz, CH-Ar), 8.02 (1 H, d, J = 7.8 Hz, Ar 6-H), 8.18 (1 H, s, Ar 2-H); MS (FAB +ve ion) *m/z* 277.1427 ($C_{16}H_{21}O$ requires 277.1440).

Prop-2-yl 2(*R*), 3(*S*)-3-acetamido-2-hydroxy-3-(3-prop-2-yloxycarbonyl)phenyl) propanoate 136



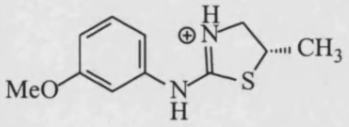
$K_2[OsO_2(OH)_4]$ (15 mg, 0.04 mmol) was dissolved in 3 ml $LiOH.H_2O$ (43 mg, 4.0 mmol). 2-Methylpropan-2-ol (6 ml) was added followed by $(DHQ)_2PHAL$ (40 mg, 0.05 mmol) and the mixture was stirred for 10 min to give a clear solution. Water (6 ml) was added and the mixture was immersed in a cooling bath 4°C. After the addition of **135** (280 mg, 1 mmol), N-bromoacetamide (152 mg, 1.1 mmol) was added in one portion and the mixture was stirred at 4°C. The reaction was monitored by TLC and pH (pH 7 indicating that the reaction was complete). After 24 h, Na_2SO_3 (5.0 g, 4.0 mmol) was added and the mixture was stirred for 30 min at 20°C. EtOAc (5 ml) was added and the organic layer was extracted, washed with brine and dried. Evaporation and chromatography (acetone/hexane 1:1) gave **126** (113 mg, 32%) as an oil; Rf 0.4 (acetone/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (6 H, d, J = 6.3 Hz, 2 × CH_3), 1.39 (6 H, d, J = 6.3 Hz, 2 × CH_3), 1.97 (3 H, s, CH_3), 4.46 (1 H, s, C*H*OH), 5.10 (1 H, m, CH), 5.24 (1 H, m, CH), 5.55 (1 H, d, J = 8.9 Hz, C*H*NH), 6.88 (1 H, d, J = 9.4 Hz, NH), 7.38 (1 H, t, J = 7.8 Hz, Ar 5-H), 7.56 (1 H, d, J = 7.8 Hz, Ar 6-H), 7.94 (1 H, d, J = 7.8 Hz, Ar 4-H), 8.06 (1 H, s, Ar 2-H); ; $[\alpha]_D^{20}$ = -12.5° (c = 13 mg ml^{-1}, MeOH);MS (FAB +ve ion) *m/z* 352.1773 ($C_{18}H_{26}NO_6$ requires 352.1760).

(*R*)-N-(2-Hydroxypropyl)-N'-(3-methoxyphenyl)thiourea 145



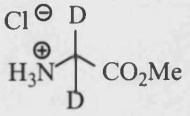
3-Methoxyisothiocyanate **(116)** (500 mg. 3.0 mmol) in acetone (2.1 ml) was added dropwise during 30 min to (R)-(-)-1-Aminopropan-2-ol (420 mg, 4.0 mmol) in acetone (2.1 ml). The mixture was boiled under reflux for 2 h. Evaporation and chromatography (EtOAc/hexane 1:1) gave **145** (270 mg, 38%) as an oil; Rf 0.3 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.22 (3 H, d, J = 6.3 Hz, CH_3), 3.46 (1 H, m, C*H*NH), 3.81 (3 H, s, OCH_3), 3.94 (1 H, m, C*H*NH), 4.02 (1 H, C*H*OH), 6.64 (1 H, br, NH), 6.78 (3 H, m, Ar 2,4,6-H_3), 7.33 (1 H, t, J = 8.2 Hz, Ar 5-H), 7.76 (1 H, br, NH); IR ν_{max} (film) 1180 (C=S), 2850 (OCH_3), 3369 (NH) cm^{-1}; $[\alpha]_D^{20}$ = -7.2° (c = 1.4 mg 1 ml^{-1}, MeOH); MS (FAB +ve ion) *m/z* 241.1007 ($C_{11}H_{17}N_2O_2S$ requires 241.1011).

(S)-2-(3-Methoxyphenylamino)-5-methyl-4,5-dihydrothiazole 146



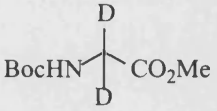
Compound **145** (80 mg, 0.3 mmol) was boiled under reflux for 24 h in aq. HCl (6 M, 4 ml). Evaporation gave **146** (70 mg, quant.) as an oil; ^{1}H NMR ($CDCl_3$) δ 1.51 (3 H, d, J = 5.9 Hz, thiazoleCH_3), 3.72 (1 H, m, C*H*NH), 3.81 (3 H, s, OMe), 3.94 (1 H, m, C*H*NH),4.11 (2 H, m, C*H*NH + CHS), 6.81 (1 H, m, Ar 6-H), 6.88 (1 H, d, J = 8.2 Hz, Ar 4-H), 7.30 (1 H, s, Ar 2-H), 7.32 (1 H, t, J = 8.2 Hz, Ar 5-H), 10.67 (1 H, br, NH), 12.29 (1 H, br, NH); IR ν_{max} (film) 1629 (C=N), 3434 (NH) cm^{-1}; $[\alpha]_D^{20}$ = -32.4° (c = 2.6 mg ml^{-1}, MeOH); MS (FAB +ve ion) *m/z* 223.08963 ($C_{11}H_{16}N_2OS$ requires 223.0905).

α,α-Dideuteroglycine methyl ester hydrochloride 148



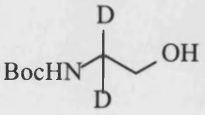
α,α-Dideuteroglycine **147** (300 mg, 3.9 mmol) was stirred with MeOH (50 ml) and $SOCl_2$ (3 ml) for 4 d. Evaporation gave **148** (460 mg, quant.) as white crystals: mp 113-115°C (lit. [Tiley *et al* 1994] mp 174-176°C; ^{1}H NMR ($(CD_3)_2SO$) δ 3.36 (3 H, s, CH_3), 8.35 (2 H, br, NH_2); IR ν_{max} (disc) 1748 (C=O), 2170 (CD_2), 3400 (NH) cm^{-1}: MS (FAB +ve ion) *m/z* 92 (M + H), 245 (M+ mNBA).

α,α-Dideutero-N-(1,1-dimethylethoxycarbonyl)glycine methyl ester 149



Di-*tert*-butyl dicarbonate (950 mg, 4.4 mmol) was added slowly to compound **148** (400 mg, 4.4 mmol) and Et_3N (730 mg, 7.2 mmol) in CH_2Cl_2 (15 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:1) gave **149** (260 mg, 31%) as an oil (lit. [Elemes and Ragnarson 1996] mp 87.5-88.5 °C); Rf 0.6 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.45 (9 H, s, Bu^t), 3.75 (3 H, s, CH_3), 5.00 (1 H, br, NH); IR ν_{max} (disc) 1747 (C=O), 2362 (CD_2), 3451 (NH) cm^{-1}: MS (FAB +ve ion) *m/z* 192 (M + H), 345 (M + mNBA).

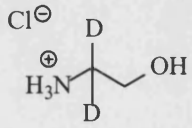
1,1-Dimethylethyl N-(1,1-dideutero-2-hydroxyethyl)carbamate 150



Compound **149** (200 mg, 1.0 mmol) was taken up in dry THF (5 ml). $LiBH_4$ (2 M solution in THF, 1.2 ml, 2.4 mmol) was added and the mixture was stirred for 16h

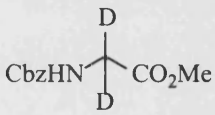
under Ar. The mixture was quenched with water and extracted with EtOAc. Evaporation and chromatography (EtOAc/hexane 1:1) gave **150** (120 mg, 73%) as an oil; Rf 0.3 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.45 (9 H, s, Bu^t), 2.44 (1 H, br, OH), 3.70 (2 H, d, J = 4.3 Hz, CH_2), 4.94 (1 H, br, NH); IR ν_{max} (film) 1716 (C=O), 2123 (CD_2), 3348 (NH) cm^{-1}: MS (FAB +ve ion) *m/z* 164.1261 (M + H) ($C_7H_{14}D_2NO_3$ requires 164.1256), 108 (M – $Me_2C{=}CH_2$).

2-Amino-2,2-dideuteroethanol 151



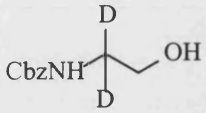
Compound **150** (100 mg, 0.6 mmol) was stirred in aq. HCl (6M, 5 ml,) for 10 min. Evaporation gave **151** (100 mg, quant.) as white crystals; mp 148-150 °C (lit. [Tiley *et al* 1994] free base bp_{10} 68-75°C); Rf 0.1 (EtOAc); ^{1}H NMR ($(CD_3)_2SO$) δ 3.57 (2 H, t, J = 5.3 Hz, CH_2), 4.21 (1 H, br, OH), 8.09 (3 H, br, NH_3^+); IR ν_{max} (disc) 2358 (CD_2), 3433 (NH); MS (FAB +ve ion) *m/z* 64 (M + H), 217 (M + mNBA).

α,α-Dideutero-N-(phenylmethoxycarbonyl)glycine methyl ester 153



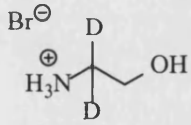
Benzyl chloroformate (380 mg, 2.2 mmol) was added slowly to **148** (200 mg, 2.2 mmol) and Et_3N (560 mg, 5.5 mmol) in CH_2Cl_2 (30 ml) at 0°C and the mixture was stirred for 16 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with aq. $NaHCO_3$ and dried. Evaporation and chromatography (EtOAc/hexane 1:1) gave **153** (220 mg, 44%) as white crystals mp 175-177°C; Rf 0.4 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 3.76 (3 H, s, CH_3), 5.13 (2 H, s, CH_2), 7.36 (6 H, m, Ph-H_5 + NH); IR ν_{max} (disc) 1666 (C=O), 2554 (CD_2), 3436 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 226.1037 ($C_{11}H_{12}D_2NO_4$ requires 226.1079), 379 (M + mNBA), 248 (M + Na).

Phenylmethyl N-(1,1-dideutero-2-hydroxyethyl)carbamate 154



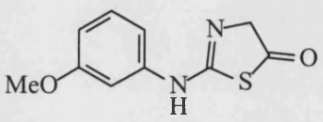
Compound **153** (210 mg, 0.9 mmol) was taken up in dry THF (2 ml). 0.1ml $LiBH_4$ was added and the mixture was stirred for 16h under Ar. The mixture was quenched with water and extracted with EtOAc. Evaporation and chromatography (EtOAc) gave **154** (150 mg, 82%) as white crystals: mp 60-62°C; (lit. [Hamada *et al* 1987] mp 60-62°C for unlabelled); Rf 0.4 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 2.80 (1 H, br, OH), 3.73 (2 H, s, CH_2OH), 5.13 (2 H, s, $PhCH_2$), 5.36 (1 H, br, NH), 7.34 (5 H, m, Ph-H_5); IR ν_{max} (film) 1668 (C=O), 2300 (CD_2), 3421 (NH + OH) cm^{-1}; MS (FAB +ve ion) *m/z* 198.1101 (M + H) ($C_{10}H_{11}D_2NO_3$ requires 198.1130).

2-Amino-2,2-dideuteroethanol bromide 155



Compound **154** (70 mg, 0.4 mmol) was stirred in HBr in HOAc (17.5%, 2 ml) for 15 h. Dry Et_2O (10 ml) was added, the mixture was stirred for 5 min and then Et_2O was decanted; this was repeated 6 times. Drying gave **155** (30 mg, quant.) as pale purple crystals: mp 62-64°C; ^{1}H NMR (CD_3OD) δ 4.28 (1 H, br, OH), 4.62 (2 H, s, CH_2); MS (FAB +ve ion) *m/z* 64 (M + H); 217 (M + mNBA).

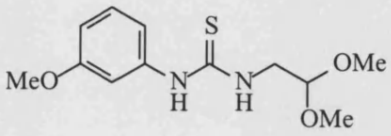
2-(3-Methoxyphenylamino)thiazole-5-one 159



Compound **116** (500 mg. 3.0 mmol) in acetone (2.1 ml) was added dropwise during 30 min to glycine ethyl ester (420 mg, 4.0 mmol) in acetone (2.1 ml). The mixture

was boiled under reflux for 16 h. Evaporation and chromatography (EtOAc/hexane 1:1) gave **159** (420 mg, 52%) as an oil; Rf 0.5 (EtOAc/hexane 1:1); ^{1}H NMR ($(CD_3)_2SO$) δ 3.78 (3 H, s, CH_3), 4.27 (2 H, s, CH_2), 6.85 (2 H, d, J = 8.2 Hz, Ar 6-H_2), 7.01 (2 H, d, J = 8.2 Hz, Ar 4-H_2), 7.35 (1 H, t, J = 8.2 Hz, Ar 5-H), 7.40 (1 H, s, Ar 2-H); ^{13}C NMR ($(CD_3)_2SO$) δ 49.6 (CH_2), 55.8 (CH_3), 114.6 (C-2), 115.3 (C-6), 121.6 (C-4), 129.9 (C-5), 135.1 (C-3), 159.9 (C-1), 172.6 (C=N), 183.8 (C=O); IR ν_{max} (film) 1694 (C=N), 1761 (C=O), 3244 (NH) cm^{-1}; MS (EI +) *m/z* 222.0464 (M) ($C_{10}H_{10}N_2O_2S$ requires 222.0463), 378 (M + mNBA).

N-(2,2-Dimethoxyethyl)-N'-(3-methoxyphenyl)thiourea 163



Compound **116** (500 mg. 3.0 mmol) in CH_2Cl_2 (2 ml) was added to 2,2-dimethoxyethylamine (280 mg, 3.0 mmol) in $CHCl_2$ (2 ml). The mixture was boiled under reflux for 1 h. Evaporation and chromatography (EtOAc/hexane 1:1) gave **163** (420 mg, 52%) as an oil; Rf 0.5 (EtOAc/hexane 1:1); ^{1}H NMR (CD_3OD) δ 3.40 (6 H, s, 2 × CH_3), 3.70 (2 H, d, J = 5.5 Hz, CH_2), 3.79 (3 H, s, CH_3), 4.61 (1 H, t, J = 5.5 Hz, CH), 6.75 (2 H, ddd, J = 0.8, 2.7, 8.5 Hz, Ar 6-H), 6.84 (2 H, ddd, J = 0.8, 2.0, 8.2 Hz, Ar 4-H), 7.01 (1 H, dd, J = 2.0, 2.7 Hz, Ar 5-H), 7.25 (1 H, t, J = 7.8 Hz, Ar 2-H); IR ν_{max} (film) 1125 (C=S), 2834 (OCH_3), 3369 (NH) cm^{-1}; MS (FAB +ve ion) *m/z* 271.1121 ($C_{12}H_{19}N_2O_3S$ requires 271.1116).

References

Abu-Soud, H. M.; Feldman, P. L.; Clark, P.; Stuehr, D. J. Electron transfer in the nitric oxide synthases. *J. Biol. Chem.* **1994**, *269*, 32318-32326.

Adock, B.; Lawson, A. 2-amino-2-imidazolines and 2-amino-2-oxazolines. Part II. *J. Chem. Soc.* **1965**, 474-479.

Alderton, W. K.; Cooper, C. E.; Knowles, R. G. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593-615.

Allescher, H.; Sattler, D.; Piller, C.; schusdziarra, V.; Classen, M. Ascending neural pathways in the rat ileum in vitro – effect of capsaicin and involvement of nitric oxide. *Eur. J. Pharmacol.* **1992**, *217*, 153-162.

Andrade, S. P.; Hart, I. R.; Piper, P. J. Inhibitors of nitric oxide synthase selectivity reduce flow in tumour-associated neovasculature. *Br. J. Pharmacol.* **1992**, *107*, 1092-1095.

Ashton, P. R.; Fyfe, M. C. T.; Hickingbottom, S. K.; Menzer, S.; Stoddart, J. F.; white, A. J. P.; Williams, D. J. Combinding different hydrogen-bonding motifs to self-assemble interwoven superstructures. *Chem. Eur. J.* **1998**, *4*, 577-589.

Atkinson, R. N.; King, B. Guanidine-subsituted imidazoles as inhibitors of nitric oxide synthase. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2953-2958.

Babu, B. R.; Frey, C.; Griffith, O. W. L-arginine binding to nitric-oxide synthase. *J. Biol. Chem.* **1999**, *274*, 2518-25226.

a Babu, B. R.; Griffth, O.W. Design of isoform-selective inhibitors of nitric oxide synthase. *Curr. Opin. Chem. Biol.* **1998**, *2*, 491-500.

b Babu, B. R.; Griffth, O.W. N^5-(1-Imino-3-butenyl)-L-ornithine. *J. Biol.Chem.* **1998**, *273*, 8882-8889.

Bachand, C.; Driguez, H.; Paton. J. M.; Touchard, D.; Lessard, J. N-monochlorination and N-monobromination of carbamates and carboxamides by sodium hypochlorite and hypobromite. *J. Org. Chem.* **1974**, *39*, 3136-3138.

Bellamy, F. D.; Ou, K. Selective reduction of aromatic nitro compounds with stannous chloride. *Tetrahedron Lett.* **1984**, *25*, 839-842.

Bredt, D. S.; Hwang, P. M.; Glatt, C. E.; Lowenstein, C.; Reed, R. R.; Snyder, S. H. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **1991**, *351*, 714-718.

Bredt, D. S.; Snyder, S. H. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci.* **1989**, *86*, 9030-9033.

Bruncko, M.; Schlingloff, G.; Sharpless, K. B. N-Bromoacetamide – a new nitrogen source for the catalytic asymmetric aminhydroxylation of olefins. *Angew. Chem. Int. Ed. Engl.*. **1997**, *36*, 1483-1486.

Bryk. R.; Wolff, D. J. Pharmacological modulation of nitric oxide synthesis by mechanism-based inactivators and related inhibitors. *Pharmacol. Therap.* **1999**, *84*, 157-178.

Buckley, N.; Oppenheimer, N. J. Reactions of charged substances. 1. The effect of product isomerization on kinetics in the reaction of thiocyanate with (4-methoxybenzyl)dimethylsulfonium chloride. *J. Org. Chem.* **1994**, 59, 247-249.

Buttery, L. D. K.; Springall, D. R.; Andrade, S. P.; Riveros-Morenos, V.; Hart, I.; Peper, P. J.; Polak, J. M. Induction of nitric oxide synthase in the neo-vasculature of experimental tumours in mice. *J. Pathol.*. **1993**, *171*, 311-319.

Calignano, A.; Whittle, B. J. R.; Rosa, M. D.; Moncada, S. Involvement of endogenous nitric oxide in the regulation of rat intestinal motility in vivo. *J. Pharmacol.* **1992**, *229*, 273-276.

Callahan, J. F.; Ashton-Shue, D.; Bryan, H. G.; Bryan, W. M.; Heckman, G. D.; Kinter, L. B.; Mc Donald, J. E.; Moore, M. L.; Schmidt, D. B.; Silvestri, J. S.; Stassen, F. L.; Sulat, L; Yim, C. F.; Huffman, W. F. Structure-activity relationships of novel vasopressin antagonists containing C-terminal diaminoalkanes and (aminoalkyl) guanidines. *J. Med. Chem.* **1989**, *32*, 391-396.

Caujoulle, R.; Amarouch, H.; Payard, M.; Loiseau, P. R.; Bories, C.; Loiseau, P. M.; Gayral, P. Aminothiazines et aminothiazoles analogues ouverts du levanisole: Synthèse et approche du mode d'action nematicide. *Eur. J. Med. Chem.* **1989**, *24*, 287-292.

Chabin, R. M.; McCauley, E.; Calaycay, J. R.; Kelly, T. M.; MacNaul, K. L.; Wolfe, G. C.; Hutchinson, N. I.; Madusudanaraju, S.; Schmidt, J. A.; Kozarich, J. W.; Wong, K. K. Active-site structure analysis of recombinant human inducible nitric oxide synthase using imidazole. *Biochemistry.* **1996**, *35*, 9567-9575.

Chavikovski, V. K; Filimonov, V. D.; Yu, A.; Kharlova, T. S. 2,4,6,8-Tetraiodglycoluril in sulphuric acid as a new powerful reagent for iodination of deactivated arenas. *Tetrahedron Lett.* **2000**, *41*, 9101-9104.

Chen, Y.; Rosazza, J. P. N. A bacterial nitric oxide synthase from a *nocardia* species. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1251-1258.

Chinje, E. C.; Stratford, I. J. Role of nitric oxide in growth of solid tumours: a balancing act. *Essays in Biochem.* **1997**, *32*, 61-72.

Cho, M. J.; Vaghy, P. L.; Kondo, R.; Lee, S. H.; Davis, J. P.; Rehi, R.; Heo, W. D.; Johnson, J. D. Reciprocol regulation of mammalian nitric oxide synthase and calcineurin by plant calmodulin isoforms. *Biochemistry.* **1998**, *37*, 15593-15597.

Christopherson, K. S.; Bredt, D.S. Perspectives series: nitric oxide and nitric oxide synthases. *J. Clin. Invest.* **1997**, *100*, 2424-2429.

Cobbs, C. S.; Brenman, J. E.; Aldpae, K. D.; Bredt, D. S.; Israel, M. A. Expression of nitric oxide synthase in human central nervous system tumours. *Cancer Res.* **1995**, *55*, 727-730.

Collins, J. L.; Shearer, B. G.; Oplinger, J. A.; Lee, S.; Garvey, E. P.; Salter, M.; Duffy, C.; Burnette, T. C.; Furfine, E. S. N-phenylamidines as selective inhibitors of human neuronal nitric oxide synthase: Structure-activity studies and demonstration of *in vivo* activity. *J. Med. Chem.* **1998**, *41*, 2858-2871.

Cooper, C. E. Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends. Biochem. Sci.* **2002**, *27*, 33-39.

Corbett, J. A.; Tilton, R. G.; Chang, K.; Hasan, K. S.; Ido, Y.; Wang, J. L.; Sweetland, M. A.; Lancaster, J. R.; Williamson, J. R.; McDaniel, M. L. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* **1992**, *41*, 552-556.

Crane, B. R.; Arvai, A. S.; Gachhui, R,; Wu, C.; Ghosh, D. K.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **1997**, *278*, 425-431.

Crane, B. R.; Arvai, A. S.; Ghosh, D. K.; Wu, C.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science.* **1998**, *279*, 2121-2126.

De Graw, J. I.; Engstrom, J. S. The synthesis of ^{14}C and ^{3}H-labeled N(2-p-azidophenylethyl)-norlevorphanol. *J. Labelled. Compd. Radiopharm* **1975**, *11*, 233-239.

Doi, K.; Akakie, T.; Hidechika, H.; Noguchi, Y.; Fujii, S.; Beppu, T.; Ogawa, M.; Maeda, H. Excessive production of nitric oxide in rat solid tumour and its implication in rapid tumour growth. *Am. Cancer Soc.* **1996**, *17*, 1598-1604.

Edwards, P.; Cendan, J. C.; Topping, D. B.; Moldawer, L. L.; MacKay, S.; Copeland, E. M.; Lind, D. S. Tumour cell nitric oxide inhibits cell growth in vitro, but stimulates tumorigenesis and experimental lung metastasis in vivo. *J. Surg. Res.* **1996**, *63*, 49-52.

Elemes, Y.; Ragnarsson, U. Synthesis of enantiopure α-deuteriated Boc-L-amino acids. *J. Chem. Soc., Perkin. Trans. 1.* **1996**, 537-540.

Emerson, W. S.; Heimsch, R. A. Some esters based on *p*-chloromethylbenzoyl chloride. *J. Am. Chem. Soc.* **1950**, 72, 5152-5154.

Ettlinger, M. G.; Lundeen, A. J. The mustard oil of *limnanthes douglasii* seed, *m*-methoxybenzyl isothiocyanate. *J. Am. Chem. Soc.* **1956**, 78, 1952-1954.

Evans, C. H.; Stefanovic-Racic, M. Nitric oxide in arthritis: it's probably there but what is it doing. *Nitric oxide, cytochromes P450 and sexual steroid hormones.* **1997**, 181-203. Eds Lancaster, J. R.; Parkinson, S. F. Springer Verlag, Berlin.

Fast, W.; Nikolic, D.; Van Breemen, R. B.; Silverman, R. B. Mechanistic studies of the inactivation of inducible nitric oxide synthase by N5-(1-iminoethyl)-L-ornithine (L-NIO). *J. Am. Chem. Soc.* **1999**, *121*, 903-916.

Folkman, J. Fighting cancer by attacking its blood supply. *Scientific American.* **1996**, 116-119.

Franks, L. M.; Teich, N. M. Introduction to the cellular and molecular biology of cancer. Second Edition. **1995**, Oxford University Press.

Frey, C.; Narayanan, K.; McMillan, K.; Spack, L.; Gross, S. S.; Masters, B. S.; Griffith, O. G. L- thiocitrulline. *J. Biol. Chem.* **1994**, *269*, 26083-26091.

Furfine, E. S.; Harmon, M. F.; Paith, J.; E.; Garvey, E. P. Selective inhibition of constitutive nitric oxide synthase by L-N^G-nitroarginine. *Biochemistry.* **1993**, *32*, 8512-8517.

Furfine, E. S.; Harmon, M. F.; Paith, J.; Knowles, R. G.; Salter, M.; Kiff, R. J.; Duffy, C.; Hazelwood, R.; Oplinger, J. A.; Garvey, E. P. Potent and selective inhibition of human nitric oxide synthase. *J. Biol.Chem.* **1993**, *269*, 26677-26683.

Garvey, E. P.; Oplinger, J. A.; Furfine, E. S.; Kiff, R. J.; Lazzlo, F.; Whittle, B. J. S.; Knowles, R. G. 1400W is a slow, tight binding, and highly selective inhibitor of iNOS in vitro and in vivo. *J. Biol. Chem.* **1997**, *272*, 4959-4963.

Garvey, E. P.; Oplinger, J. A.; Tonoury, G. J.; Sherman, P. A.; Fowler, M.; Marshall, S,; Harmon, M. F.; Paith, J. E.; Furfine, E. S. Potent and selective inhibition of human nitric oxide synthase. *J. Biol. Chem.* **1994**, *269*, 26669-26676.

Gatto, E. M.; Riobo, N. A.; Carreras, M. C.; Chernavsky, A.; Rubio, A.; Satz, M. L.; Poderosos, J. J. Overexpression of neutrophil neuronal nitric oxide synthase in parkinson's disease. *Nitric Oxide* **2000**, *4*, 534-539.

Giordano, C.; Castaldi, G.; Casagrande, F.; Belli, A. Esters of α-arylalkanoic acids from 'masked' α-halogenoalkyl aryl ketones and silver salts: synthetic, kinetic and mechanistic aspects. *J. Chem. Soc., Perkin. Trans.1.* **1982**, 2575-2581.

Griffith, O.W.; Steuhr, D. J. Nitric oxide synthases: properties and catalytic mechanism. *Ann. Rev. Physiol.* **1995**, *57*, 707-736.

Gross, S. S. Targeted delivery of nitric oxide. *Nature* **2001**, *409*, 577-578.

Gross, S. S.; Wolin, M. S. Nitric oxide: pathophysiological mechanisms. *Ann. Rev. Physiol.* **1995**, *57*, 737-769.

Hallinan, E. A.; Tsymbalov, S.; Finnegan, P. M.; Moore, W. M.; Jerome, G. M.; Currie, M. G.; Pitzele, B. S. Acetamidine lysine derivatives, N-(5(S)-amino-6,7-dihydroxyheptyl)ethanimidamide dihydrochloride: a highly selective inhibitor of human inducible nitric oxide synthase. *J. Med. Chem.* **1998**, *41*, 775-777.

Hamada, Y.; Shibata, M.; Sugiura, T.; Kato, T.; Shioiri, T. New methods and reagents in organic synthesis. A general synthesis of derivatives of optically pure 2-(1-Aminoalkyl)thiazole-4-carboxylic acids. *J. Org. Chem.* **1987**, *52*, 1252-1255.

Hellman, S.; Vokes, E. E. Advancing current treatments for cancer. *Scientific American* **1996**, 85-87.

Hevel, J. M.; White, K. A.; Marletta, M. Purification of the inducible murine macrophage nitric oxide synthase. *J. Biol. Chem.* **1991**, *266*, 22789-22791.

Hirashima, A.; Yoski, Y.; Eto, M. Synthesis and octopaminergic agonist activity of 2-(substituted benzylamino)–2-thiazolines. *Biosci. Biotech. Biochem.* **1992**, *56*, 1062-1065.

Hughes, D. K. The mitsunobu reaction. *Organic reactions*, **1992**, *42*, 337-636. Eds. Paquette, L. A.; Beak, P.; Ciganek, E.; Curran, D.; Hegedus, L.; Overman, L. E.; Roush, w.; Sih, C.; Smith, A. B.; uskokovic, M.; White, J. D. Wiley. New York.

Hűter, F. Uber die Darstellung von substituierten Thioharnstoffen. *Chem. Ber.* **1947**, *3*, 273-277.

Ialenti, A.; Moncada, S.; Rosa, M. D. Modulation of adjuvant arthritis by endogenous nitric oxide. *Br. J. Pharmacol.* **1993**, *10*, 701-706.

Ignarro, L. J. Biosynthesis and metabolism of endotheliaium-derived nitric oxide. *Ann. Rev. Pharm. Toxicol.* **1990**, *30*, 535-560.

Jacobs, W. A.; Heidelberger, M. Amides of aminophenylacetic acids. *J. Am. Chem. Soc.* **1917**, 39, 2420.

Jenkins, D. C.; Charles, I. G.; Thomsen, L. L.; Moss, D. W.; Holmes, L. S.; Baylis, S. A.; Rhodes, P.; Westmore, K.; Emson, P. C.; Moncada, S. Roles of nitric oxide in tumour growth. *Proc. Natl. Acad. Sci.* **1995**, *92*, 4392-4396.

Kerwin, J. F.; Lancaster, J. R.; Feldman, P. L. Nitric oxide: A new paradigm for second messengers. *J. Med. Chem.* **1995**, *32*, 4343-4362.

Kilbourn, R. G.; Jubran, A.; Gross, S. G.; Griffth, O. W.; Levi, R.; Adams, J.; Lodalo, R. F. Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1132-1138.

Klatt, P.; Schmidt, K.; Brunner, F.; Mayer, B.; Inhibitors of brain nitric oxide synthase. *J. Biol. Chem.* **1994**, *269*, 1674-1680.

Knowles, R. G.; Merrett, M.; Salter, M.; Moncada, S. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.* **1990**, *270*, 833-836.

Knowles, R.G.; Moncada, S. Nitric oxide synthases in mammals. *Biochem. J.* **1994**, *298*, 249-258.

Kocienski, P. J. Protecting Groups. **1994**, Georg Thieme Verlag, Stuttgart.

Korth, H.; Sustmann, R.; Thater, C.; Butler, A. R.; Ingold, K. U. On the mechanism of the nitric oxide synthase catalysed conversion of N^{ω}-hydroxy-L-arginine to citrulline and nitric oxide. *J. Biol. Chem.* **1994**, *269*, 17776-17779.

Kumar, V. B.; Bernardo, A. E.; Buddhiraju, A. M.; Prushothaman, R.; Morley, J. E. Rapid assay for nitric oxide synthase using thin-layer chromatography. *Anal. Biochemistry.* **1999**, *269*, 17-20.

Lala, P. K.; Orucevic, A. Role of nitric oxide in tumour progression: lessons from experimental tumours. *Cancer Metastasis Rev.* **1998**, *17*, 91-106.

Lee, K. H.; Baek, M. Y.; Moon, K. Y.; Song, W. K.; Chung, C. H.; Ha, D. B.; Kang, M. Nitric oxide as a messenger molecule for myoblast fusion. *J. Biol. Chem.* **1994**, *269*, 14371-14374.

Lee, Y.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. Imidazole-containing amino acids as selective inhibitors of nitric oxide synthases. *Bioorg. Med. Chem.* **1999**, *7*, 1941-1951.

Lee, Y.; Martasek, P.; Roman, L. J.; Silverman, R. B. 1*H*-pyrazole-1-carboxamidines: new inhibitors of nitric oxide synthase. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2771-2774

Li, H.; Raman, C. S.; Martasek, P.; Masters, B. S. S.; Poulos, T. Crystallographic studies on endothelial nitric oxide synthase complexed with nitric oxide and mechanism-based inhibitors. *Biochemistry*. **2001**, *40*, 5399-5406.

Liu, Y.; Zhoa, C.; Bergbreiter, D. E.; Romo, D. Simultaneous deprotection and purification of Boc-amines based on ionic resin capture. *J. Org. Chem.* **1998**, 63, 3471-3473.

Marletta, M. A. Nitric oxide synthase structure and mechanism. *J. Biol .Chem.* **1993**, *268*, 12231-12234.

a Marletta, M. A. Approaches toward selective inhibition of nitric oxide synthase. *J. Med. Chem.* **1994**, *37*, 1899-1907.

b Marletta, M. A. Nitric oxide synthase: Aspects concerning structure and catalysis. *Cell.* **1994**, *78*, 927-930.

Martasek, P.; Liu, Q.; Liu, J.; Roman, L. J.; Gross, S. S.; Sessa, W. C.; Masters, B. S. S. Characterisation of bovine endothelial nitric oxide synthase expressed in *E. coli*. *Biochem. Biophys. Res. Commun.m.* **1996**, *219*, 359-365.

Maxwell, A. J. Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. *Nitric Oxide* **2002**, *6*, 101-124.

McDaniel, M. L.; Kwon, G.; Hill, J. R.; Marshall, C. A.; Corbett, J. A. Cytokines and nitric oxide in islet inflammation and diabetes. *Proc. Soc. Exp. Biol. Med.* **1996**, *211*, 24-32.

McKay, A. F.; Whittingham, D. J.; Keeling, M. E. Amino Acids VIII. 2-thiazoline and Δ^2- dihydro-1,3-thiazine derivatives of ω-amino acids. *J. Am. Chem. Soc.* **1958**, 3330-3341.

Metzger, J. V. Comprehensive heterocyclic chemistry. Eds Katritzky, A. R.; Rees, C. W. **1984**. Pergamon Press. Oxford.

Meyer, R. E.; DeAngelo, J.; Dodge, R. K.; Bonaventura, J.; Ong, E. T.; Dewhirst, M. W. Nitric oxide synthase inhibition irreversibly decreases perfusion in the R3230Ac rat mammary adenocarcinoma. *Br. J. Cancer.* **1995**, *71*, 1169-1174.

Milkiewicz, K. L.; Marsilje, T. H.; Woodworth, R. P.; Bifulco, N.; Hangauer, M. J.; Hangauer, D. G. The design, synthesis and activity of non-ATP competitive inhibitors of $pp60^{c\text{-}src}$ tyrosine kinase. Part 2: hydroxyindole derivatives. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 483-486.

Molina, P.; Alajarin, M.; Arques, A. Convenient improved syntheses of isocyanates or isothiocyanates from amines. *Synthesis* **1982**, *596-597.*

Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **1991**, 43, 109-142.

Moore, W. M.; Webber, R. K.; Fok, K. F.; Jerome, G. M.; Kornmeier, C. M.; Tjoeng, F. S.; Currie, M, G. Inhibitors of human nitric oxide synthase isoforms with the carbamidine moiety as a common structural element. *Bioorg. Med. Chem.* **1996**, *4*, 1559-1564.

Moore, W. M.; Webber, R. K.; Jerome, G. M.; Tjoeng, F. S.; Misko, T. P.; Currie, M. G. L-N^G-(1-iminoethyl)lysine: A selective inhibitor of inducible nitric oxide synthase. *J. Med. Chem.* **1994**, *37*, 3886-3888.

Muller, U.; Bicker, G. Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthesising neurons in the nervous system of the locust. *J. Neurosci.* **1994**, *14*, 7521-7528.

Nakane, N.; Klinghofer, V.; Kuk, J. E.; Donnelly, J. L.; Budzik, G. P.; Pollock, J. S.; Basha, F.; Carter, G. W. Novel potent and selective inhibitors of inducible nitric oxide synthase. *Mol. Pharmacol.* **1995**, *47*, 831-834.

Napoli, C.; Ignarro, L. J. Nitric oxide and atherosclerosis. *Nitric Oxide* **2001**, *5*, 88-97.

Narayanan, K.; Griffith, O. W. Synthesis of L-thiocitrulline, L-homothiocitrulline, and S-methylthiocitrulline: A new class of potent nitric oxide synthase inhibitors. *J. Med. Chem.* **1994**, *37*, 885-887.

Narayanan, K.; Spack, L.; McMillan, K,; Kilbourn, R. G.; Hayward, M. A.; Masters, B. S. S.; Griffith, O. G. S-alkyl-L-thiocitrullines. *J. Biol. Chem.* **1995**, 270, 11103-11110.

Nathan. C. Nitric oxide as a secretory product of mammalian cells. *FASEB. J.* **1992**, *6*, 3051-3064.

Nathan, C. Perspectivies series: nitric oxide and nitric oxide synthase. *J. Clin. Invest.* **1997**, *100*, 2417-2423.

Nathan, C.; Xie, Q. Regulation of biosynthesis of nitric oxide. *J. Biol.Chem.* **1994**, *269*, 13725-13728.

Ogden, J. E.; Moore, P. K. Inhibition of nitric oxide synthase – potential for a novel class of therapeutic agent? *Trends Biotech.* **1995**, *13*, 70-78.

Padron, J. M.; Martin, V. S.; Hadjipavlou-Litina, D.; Noula, C,; Constantinou-Kokotou, V.; Peters, G. J.; Kokotos, G. Synthesis, in vitro cytotoxicity and in vivo

anti-inflammatory activity of long chain 3-amino-1,2-diols. *Bioorg. Med. Chem. Lett.* **1999,** *9*, 821-826.

Palmer, R. M. J.; Ferringe, A. G.; Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **1987**, *327*, 524-526.

Perry, J. M.; Zhao, Y.; Marletta, M.A. Cu^{2+} and Zn^{2+} inhibit nitric-oxide synthase through an interaction with the reductase domain. *J. Biol. Chem.* **2000**, *275*, 14070-14076.

Pfeiffer, S.; Leopold, E.; Schmidt, K.; Brunner, F.; Mayer, B. Inhibition of nitric oxide synthesis by N^G-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, N^G-nitro-L-arginine. *Br. J. Pharmacol.* **1996**, *118*, 1433-1440.

Pfeiffer, S.; Mayer, B.; Hemmens, B. Nitric oxide: Chemical puzzles posed by a biological messenger. *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 1714-1731.

Physiology or Medicine for 1998 Press Release. Press release: the 1998 Noble Prize in physiology or medicine. [www] URL: http:/wwww.nobel.se/medicine/laureates/1998/press.html [Assessed 23 April 2002].

Poulos, T. L.; Raman, C. S.; Li, H. NO news is good news. *Structure* **1998**, *6*, 255-258.

Ranganathan, D.; Patel, B. K.; Mishra, R. K. Design of a chemical nuclease model with $(Lys)_2Cu$ as the core motif. *J. Chem. Soc., Chem. Comm.* **1993**, *4*, 337-339.

Rasmussen, C. R.; Villani, F. J.; Weaner, L. E.; Reynolds, B. E.; Hood, A. R.; Hecker, L. R.; Nortey, S. O.; Hanslin, A.; Costanzo, M. J.; Powell, E. T.; Molinari, A. J. Improved procedures for the preparation of cycloalkyl-, arylalkyl-, and arylthioureas. *Synthesis* **1988**, 456-459.

Rees, D. D; Palmer, R. M. J; Schulz, R; Hodson, H. F; Moncada, S. Characterisation of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.* **1990**, *101*, 746-752.

Richter, P.; Wagner, G.; Michalke, B.; Schwab, M. Synthese von 4-Guanidinophenylbrenztraubensaure und anderen Guanidinophenylalkylcarbonsauren. *Pharmazie* **1974**, *29*, 307-312.

Salerno, L.; Sorrenti, V.; Giacomo, C, D.; Romeo, G.; Siracusa, M. A. Progress in the development of selective nitric oxide synthase (NOS) inhibitors. *Curr. Pharm. Design* **2002**, *8*, 177-200.

Salter, M.; Knowles, R. G.; Moncada, S. Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.* **1991**, *291*, 145-149.

Schmidt, H. H. H. W.; Walter, U. NO at work. *Cell* **1994**, *78*, 919-925.

Schumann, P.; Collot, V.; Hommet, Y.; Gsell, w.; Dauphin, F.; Sopkova, J.; MacKenzie, E. T.; Duval, D.; Boulouard, M.; Rault, S. Inhibition of neuronal nitric oxide synthase by 7-methoxyindazole and related substituted indazoles. *Bioorg. Med. Chem.* **2001**, *11*, 1153-1156.

Shearer, B. G.; Lee, S.; Oplinger, J. A.; Flick, L. w.; Garvey, E. P.; Furfine, E. S. Substituted N-phenylisothioureas: potent inhibitors of human nitric oxide synthase with neuronal isoform selectivity. *J. Med. Chem.* **1997**, *40*, 1901-1905.

Shepherd, G. M. Neurobiology 3rd Edition. **1994**, Oxford University Press. Oxford.

Shridhar, D. R.; Sastry, C. V. R.; Gurumurthy, B. L. R.; Parihar, P.; Singh, P. P.; Naidu, M. U. R.; Seshagiri Rao, C.; Junnarkar, A. Y. Synthesis and pharmacology of some new methyl 4-[2-alkylthio-4-(3*H*)-quinazolon-3-yl]phenyl acetates. *J. Indian Chem. Soc.* **1985**, *62*, 687-689.

Siddhanta, U.; Wu, C.; Abu-Soud, H. M.; Zhang, J.; Ghosh, D. K.; Stuehr, D. J. Heme Iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. *J. Biol. Chem.* **1996**, *271*, 7309-7312.

Smith, J.; Liras, J. L.; Schneider, E.; Anslyn, E. V. Solid and solution phase organic syntheses of oligomeric thioureas. *J. Org. Chem.* **1996**, *61*, 8811-8818.

Snyder, S. H.; Bredt, D. S. Biological roles of nitric oxide. *Scientific Amer*ican **1992**. 28-35.

Sorrenti, V.; Giacomo, D.; Salerno, L.; Siracusa, M. A.; Guerrera, F.; Vanella, A. Inhibition of neuronal nitric oxide synthase by N-phenacyl imidazoles. *Nitric Oxide* **2001**, *5*, 32-38.

Southan, G. J.; Gauld, D.; Lubeskie, A.; Zingarelli, B.; Cuzzocrea, S.; Salzman, A. L.; Szabo, c.; Wolff, D. J. Inhibition of nitric oxide synthase with pyrazole-1-carboxamidine and related compounds. *Biochem. Pharmacol.* **1997**, *54*, 409-417.

Southan, G. J.; Szabo, C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* **1996**, *51*, 383-394.

Stamler, J. S. Redox signalling: Nitrosylation and related target interactions of nitric oxide. *Cell* **1994**, *78*, 931-936.

Stamler, J. S.; Singel, D. J.; Loscalzo, J. Biochemistry of nitric oxide and its redox-activated forms. *Science* **1992**, *258*, 1898-1902.

Stuehr, D. J.; Kwon, N. S.; Nathan, C. F.; Griffth, O.W. N^{ω}-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* **1991**, *266*, 6259-6263.

Stokker, G. E.; Deana. A. A.; deSolms, S. J.; Schultz, E. M.; Smith, R. L.; Cragoe, E. J.; Baer, J. E.; Russo, H. F.; Watson, L. S. 2-(aminomethyl)phenols, a new class of

saluretic agents. 4. Effects of oxygen and/or nitrogen substitution. *J. Med. Chem.* **1982**, *25*, 735-742.

Sutherland, R. M.; Rasey, J. S.; Hill, R. P. Tumour Biology. *Am. J. Clin. Oncol.* **1988**, *11*, 253-274.

Szabo, C.; Dawson, V. L. Role of poly(ADP-ribose)synthetase in inflammation and ischemia-reperfusion. *TiPS.* **1998**, *19*, 287-298.

Szabo, C.; Ohshima, H. DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* **1997**, *1*, 373-385.

Tanaka, A.; Terasawa, T.; Hagihara, H.; Sakuma, Y.; Ishibe, N.; Sawada, M.; Takasugi, H.; Tanaka, H. Inhibitors of acyl-CoA:cholesterol O-acyltransferase. 2. Identification and structure-activity relationships of a novel series of N-alkyl-N-(heteroaryl-substituted benzyl)-N-arylureas. *J. Med. Chem.* **1998**, *41*, 2390-2410.

Tanaka, K.; Yoshifuji, S.; Nitta, Y. A new method for the synthesis of amides from amines: ruthenium tetroxide oxidation of N-protected alkylamines. *Chem. Pharm. Bull.* **1988**, *36*, 3125-3129.

Thomas, G. Medicinal chemistry (an introduction). **2000**, Wiley, Chichester.

Thomsen, L. L.; Lawton, F. G.; Knowles, R. G.; Beesley, J. E.; Riveros-Moreno, V.; Moncada, S. Nitric oxide synthase activity in human gynecological cancer. *Cancer Res.* **1994**, *54*, 1352-1354.

Thomsen, L. L.; Miles, D. W.; Happerfield, L.; Bobrow, L. G.; Knowles, R. G.; Moncada, S. Nitric oxide synthase activity in human breast cancer. *Br. J. Cancer* **1995**, *72*, 41-44.

Thomsen, L. L.; Scott, J. M. J.; Topley, P.; Knowles, R. G.; Keerie, A.; Frend, A. J. Selective inhibition of iNOS inhibits tumour growth *in vivo*: Studies with 1400W, a novel inhibitor. *Cancer Res.* **1997**, *57*, 3300-3304.

Tiley, K.; Akhtar, M.; Gani, D. The sterochemical course of decarboxylation, transamination and elimination reactions catalysed by *Escherichia coli* glutamic acid decarboxylase. *J. Chem. Soc., Perkin. Trans. 1* **1994**, 3079-3087.

Tisler, M. Beitrag zur Synthese und Vmlagerung 1-substituierter Thiocarbamylathylenimine in N-substituierte Derivate des 2-Amino-2-thiazolins. *Arch. Pharm.* **1958**, *9*, 457-463.

Tozer, G. M.; Prise, V. E.; Chaplin, D. J. Inhibition of nitric oxide synthase induces a selective reduction in tumour blood flow that is reversible with L-arginine. Cancer Res. **1997**, *57*, 948-955.

Ulhaq, S.; Chinje, E. C.; Naylor, M. A.; Jaffar, M.; Stratford, I. J.; Threadgill, M. D. Heterocyclic analogues of L-citrulline as inhibitors of the isoforms of nitric oxide synthase (NOS) and identification of N^{δ}-(4,5-dihydrothiazol-2-yl)ornithine as a potent inhibitor. *Bioorg. Med. Chem.* **1999**, *7*, 1787-1796.

Ulhaq, S.; Chinje, E. C.; Naylor, M. A.; Jaffar, M.; Stratford, I. J.; Threadgill, M. D. *S*-2-amino-5-azolylpentanoic acids related to L-ornithine as inhibitors of the isoforms of nitric oxide synthase (NOS). *Bioorg. Med. Chem.* **1998**, *6*, 2139-2149.

Ware, R. W.; King, B. Evaluation of new L-thiocitrulline derivatives as inhibitors of nitric oxide synthase. *Bioorg. Med. Chem.* **2000**, *10*, 2779-2781.

Watson, C. Y. PhD thesis. University of Bath. **1997**.

Webb, P.; Threadgill, M. D. Labelled compounds of interest as antitumour agents 3. synthesis of ^{2}H and ^{3}H isotopomers of etanidazole (SR 2508). *J. Labelled compd. Radiopharm.* **1990**, *28*, 265-271.

Webber, R. K; Metz, S; Moore, W. M; Connor, J. R; Currie, M. G; Fok, K. F; Hagen, T. J; Hansen, D. W; Jerome, G. M; Manning, P. T; Titzele, B. S; Toth, M. V; Trivedi, M; Zupec, M. E; Tjoeng, F. S. Subsituted 2-iminopiperidines as inhibitors of human nitric oxide synthase isoforms. *J. Med. Chem.* **1998**, *41*, 96-101.

Weinberg, R. A. How cancer arises. *Scientific American* **1996,** 32-40.

White, K. A.; Marletta, M. A. Nitric oxide is a cyctochrome P-450 type hemoprotein. *Biochemistry.* **1992,** *31,* 6627-6631.

Williams, D. H.; Fleming, I. Spectroscopic methods in organic chemistry third edition. **1980,** McGraw-Hill, London.

a Wink, D. A.; Hanbauer, I.; Grisham, M. B.; Laval, F.; Nims, R. W.; Laval, J.; Cook, J.; Pacelli, R.; Liebman, J.; Krishna, M.; Ford, P. C.; Mitchell, J. B. Chemical biology of nitric oxide: Regulation and protective and toxic mechanisms. *Curr. Topics Cell. Reg.* **1996,** *34,* 159-187.

b Wink, D. A.; Hanbauer, I.; Grisham, M. B.; Mitchell, J. B.; Ford, P. C. Direct and indirect effects of nitric oxide in chemical reactions relevant to biology. *Methods in Enzymol.* **1996,** *268,* 12-31.

Wink, D. A.; Vodovotz, Y.; Cook, J.A.; Krishna, M. C.; Kim, S.; Coffin, D.; DeGraff, W.; Deluca, A. M.; Liebmann, J.; Mitchell, J. B. The role of nitric oxide chemistry in cancer treatment. *Biochemistry. (Moscow)* **1998,** 63, 802-809.

Wood, P. J.; Stratford, I. J.; Adams, G. E.; Szabo, C.; Thiemermann, C.; Vane,J. R.; Modifications of energy metabolism and radiation response of a murine tumour by changes in nitric oxide availability. *Biochem. Biophys. Res. Commun..* **1993,** *192,* 505-510.

Wolff, D. J.; Datto, G. A.; Samatovicz, R. A.; Tempsick, R. A. Calmodulin-dependent nitric-oxide synthase. *J. Biol. Chem.* **1993,** *268,* 9425-9429.

Wolff, D. J; Gribin, B. J. The inhibition of the constitutive and inducible nitric oxide synthase isoforms by indazole agents. *Arch. Biochem. Biophys.* **1994,** *311,* 300-306.

Wolff, D. J.; Lubeskie, A. Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch. Biochem. Biophys.* **1995**, *316*, 290-301.

Wolff, D. J.; Lubeskie, A.; Gauld, D. S.; Neulander, M. J. Inactivation of nitric oxide synthases and cellular nitric oxide formation by N^6-iminoethyl-L-Lysine and N^5-iminoethyl-L-ornithine. *Eur. J. Pharmacol.* **1998**, *350*, 325-334.

Wolff, D. J.; Naddelman, R. A.; Lubeskie, A.; Saks, D. A. Inhibition of nitric oxide synthase isoforms by porphyrins. *Arch. Biochem. Biophys.* **1996**, *333*, 27-34.

Zhang, H. Q; Fast, W; Marletta, M. A; Martasek, P; Silverman, R. B. Potent and selective inhibition of neuronal nitric oxide synthase by N^{ω}-propyl-L-arginine. *J. Med. Chem.* **1997**, *40*, 3869-3870.

Pharmacological actions of two NOS inhibitors: N-(3-aminomethyl)phenylmethylthiourea and L-NAME on *in vitro* longitudinal muscle of rat ileum

Introduction

Nitric oxide has been found to be involved in the relaxation of smooth muscle. Evidence comes from *in vitro* studies which show that nitric oxide is involved in the nonadrenergic-noncholinergic response in circular and longitudal muscle of rat ileum and causes an increase in cGMP which leads to a relaxation of smooth muscle (Allescher *et al* 1992). Ninety percent of the total NOS found in rat ileum is nNOS and iNOS, with iNOS being predominant.

L-NAME has been shown to cause frequent intestinal muscular contractions in rats when administered intravenously. These contractions were inhibited by L-arginine and therefore suggest NOS inhibition plays a role in this response. The constriction of vascular smooth muscle is thought to occur by inhibition of nNOS while contraction of the gastrointestinal tract occurs through inhibition of nNOS and iNOS.

Aim

The aim of this study was to evaluate the pharmacological effects of N-(3-aminomethyl)phenylmethyl)thiourea (**35**) on longitudinal muscle in isolated rat ileum, compared to the known NOS inhibitor L-NAME (**3**).

Methods

These experiments were carried out by MPharm undergraduate project students Laura Baber and Georgina Watson.

Preparation of ileal segments

Male Wistar rats were killed and the ileum was dissected into approximately 3 cm segments which were washed with Tyrode solution and suspended vertically in their longitudinal direction at a load of 1 g in a 30 ml organ bath. The organ bath contained Tyrode solution at 37°C and gassed with 5% CO_2 in O_2. The tissues were

allowed to equilibrate for 30 min. Initial organ bath concentrations used for the cumulative dose response curves for **35** and **3** were 1×10^{-8} M.

Recording of response of ileal longitudinal muscle

The activity of the longitudinal muscle of the rat ileum segments was recorded on isotonic transducers and the data was transferred and recorded on the computer software program, Powerlab. Relaxation was defined as the lengthening of the preparation and was recorded on Powerlab as a decrease in voltage. Contraction was defined as shortening of the preparation and was recorded on Powerlab as an increase in voltage.

Results

Compounds **3**, **35** and isoprenaline all caused the longitudinal muscle of rat ileum segments to relax. EC_{50} values could not be obtained for the NOS inhibitors, as there was a limited supply of both inhibitors. Therefore threshold values at which the compounds elicited a relaxation response were calculated.

Compound added to rat ileum in organ bath	N	Mean threshold value (Molar)	Standard deviation
35	20	1.6×10^{-5}	2.5×10^{-5}
3	8	3.1×10^{-5}	3.4×10^{-5}
Isoprenaline	14	4.2×10^{-8}	5.1×10^{-8}

Table 7.1: Mean threshold values for NOS inhibitors **35** and **3**.

The table above shows the mean threshold values obtained. Compound **35** required the highest concentration to elicit a response; however, there was no statistical

difference between **35** and **3** but, there were statistically significant differences between isoprenaline and **3** and isoprenaline and **35**. Figure 5.6 shows the relaxation curves for compounds **35** and **3**.

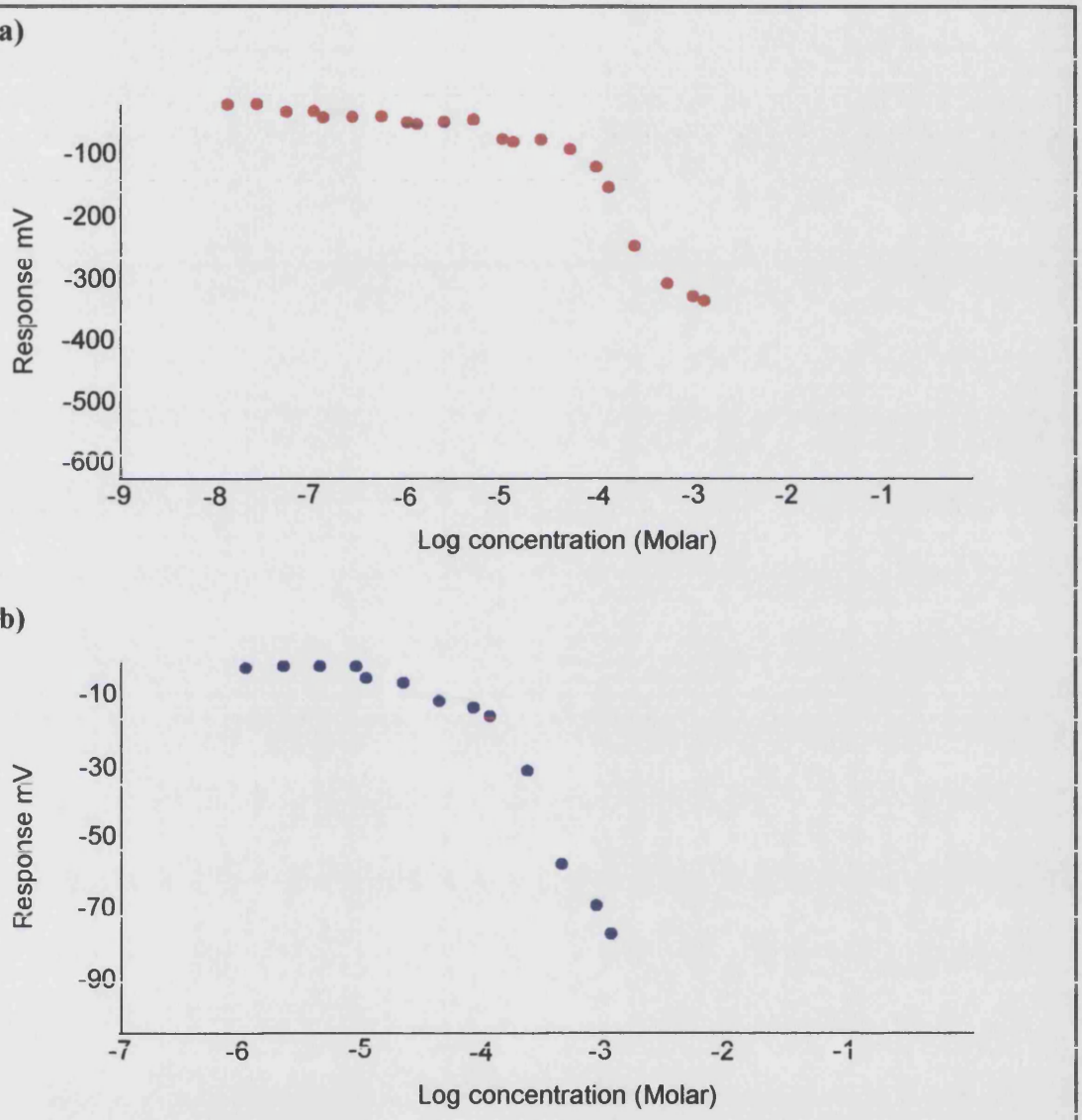


Figure 7.1: shows a) response (relaxation) of pre-contracted rat ileum following the addition of **35**; b) response (relaxation) of pre-contracted rat ileum following the addition of **3**.

Conclusions

The two NOS inhibitors used in this study, **3** and **35**, elicited a relaxation response in pre-contracted rat ileum. These data conflicts with other studies involving **3**, (Allescher *et al* 1992 and Calignano *et al* 1992) in which the compound has been shown to cause a contraction both *in vitro* and *in vivo*. However, the methods used in previous studies differ from those used in this study.

The differences in the methods used may explain why a relaxation response was observed and not a contractile response. The NOS enzymes were not activated in the resting smooth muscle cells and, therefore, inhibition of the NOS enzymes with **3** or **35** did not elicit a contractile response in the rat ileum. Alternatively, if NOS enzymes were active, a contractile response may not have been observed because **3** and **35** were not allowed sufficient time to equilibrate in order to inhibit the NOS enzymes.

This study has shown that both NOS inhibitors **3** and **35** can elicit a relaxation response that is unrelated to their inhibitory action of the NOS enzymes. The mechanism by which this relaxation occurs remains unknown.

Published Work

Claire L. M. Goodyer, Michael D. Threadgill, Edwin C. Chinje, Mohammed Jaffar,
Ian J. Stratford

Thioureas and 4,5-dihydrothiazoles as inhibitors of the isoforms of nitric oxide synthase for cancer therapy

Nitric Oxide, **2002**, *6*, 393.

Thioureas and 4,5-dihydrothiazoles as inhibitors of the isoforms of nitric oxide synthase for cancer therapy

Claire L. M. Goodyer, [a] Michael D. Threadgill, [a] Edwin C. Chinje, [b] Mohammed Jaffar, [b] Ian J. Stratford [b]

[a] *Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK*

[b] *MRC Experimental Oncology Laboratory, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PL, UK*

Human tumours have high levels of NO, the main source deriving from iNOS. NO is responsible for the increase in blood flow to the tumour. The administration of the iNOS inhibitor 1400W (**1**) has been shown to reduce the rate of solid tumour growth in mice (Thomsen *et al* 1997). Selective inhibition of tumours should cause collapse of the tumour vasculature, which may be an antitumour event in itself but which will also increase tumour hypoxia and potentiate the cytotoxicity of bioreductively activated drugs.

N^{δ}-(4,5-dihydrothiazol-2-yl)-L-ornithine (**2**), *S*-2-amino-5-(imidazol-1-yl)pentanoic acid (**3**) and (**1**) have been used as lead compounds in the design of potent and selective NOS inhibitors. Both **2** and **3** were potent but not isoform-selective; **2** has IC_{50} values of 32 μM, 19 μM and 13 μM for iNOS, nNOS and cNOS, respectively. Compound **3** has IC_{50} values of 8 μM, 4 μM and 1 μM for iNOS, nNOS and cNOS, respectively. New compounds have been designed using the potent thiazole head group and incorporating features of the selective iNOS inhibitor **1** (Ki 0.14 μM iNOS) with the aim of giving rise to both potent and selective inhibitors.

N-(3-Aminomethyl)phenylmethyl)thiourea (**4**) was synthesised from 3-nitrobenzylamine. Boc protection and reduction of the nitro group, followed by treatment with thiophosgene, gave the isothiocyanate. Reaction with ammonia gave the thiourea and deprotection with trifluoroacetic acid gave the target. The 2,4-dihydrothiazole head group was added to methoxybenzylamine under harsh conditions, (180°C, 4 h) using 2-methylthio-4,5-dihydrothiazole. Other structurally related targets were synthesised analogously. All compounds were initially screened at one concentration (100 μM) in the presence of 10 μM arginine, for inhibition of rat brain cNOS and rat lung iNOS; results below are expressed as % inhibition. Wherever preliminary screening results showed good inhibition, the IC_{50} values were determined.

Compound **4** was the most potent inhibitor against iNOS and cNOS. (IC_{50} 30 μM and 6 μM for iNOS and cNOS, respectively). The compounds tested showed limited isoform selectivity, of which the greatest selectivity for iNOS was seen for **5** (iNOS 55%, cNOS 25%) and **6** (iNOS 46%, cNOS 21%) after five minutes pre-incubation. In contrast, **4** showed the greatest selectivity for cNOS (iNOS 27%, cNOS 88%), especially after a five minute pre-incubation. The selectivity for iNOS in **1** is thought to be based on the 7-atom bridge between the remote Fe-binding amidine N and the $N^{+}H_3$. The 4,5-dihydrothiazole and thiourea head groups may be important in the potency of the inhibitor. The pre-incubation time appears to be important in the isoform selectivity of the compounds, suggesting that slow-binding inhibition may be involved.